CHOLINE METABOLISM IN RESPONSE TO CHOLINE INTAKE, PREGNANCY, AND POLYMORPHISMS OF ONE-CARBON METABOLIC GENES IN HUMANS

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CHOLINE METABOLISM IN RESPONSE TO CHOLINE INTAKE, PREGNANCY, AND POLYMORPHISMS OF ONE-CARBON METABOLIC GENES IN HUMANS

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Choline, an essential nutrient, is critical in maintaining biomembrane integrity (via phosphatidylcholine) and supplying methyl groups for one-carbon metabolism (via betaine). Choline adequate intakes were established for the first time in 1998. However, little is known about the impact of pregnancy and genetic variation on choline metabolism and requirements. The overall goal of my research was to quantify the effects of pregnancy, genetic variation, and choline intake on biomarkers of choline metabolism.

To achieve this goal, two separate feeding studies that employed stable isotope methodology were conducted. Study 1 examined the effect of the methylenetetrahydrofolate reductase (*MTHFR*) 677C→T genetic variant, and choline intake, on biomarkers of choline metabolism. Study 2 investigated the effect of pregnancy, and choline intake, on biomarkers of choline metabolism. Deuterium labeled *methyl*-d9-choline was administered in both studies as the tracer.

Study 1 demonstrated that the *MTHFR* 677TT (versus the 677CC) genotype favors the use of choline as a methyl donor. *MTHFR* 677TT genotype enhanced the conversion of choline to betaine. In addition, when a higher choline intake was consumed, more of the choline was converted to betaine as opposed to entering the CDP-choline pathway for phosphatidylcholine synthesis among men with *MTHFR* 677TT (versus 677CC) genotype.

Study 2 demonstrated that pregnancy alters choline metabolism with 10-60% lower circulating concentrations of choline derived methyl donors among pregnant versus nonpregnant women. Stable isotope data suggested that pregnancy increased choline partitioning to the CDP-choline pathway at the expense of betaine synthesis, and also increased the use of choline-derived methyl groups for methionine synthesis and phosphatidylcholine synthesis through the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway. Despite the upregulation of both pathways for phosphatidylcholine synthesis, PEMT-phosphatidylcholine was selectively transferred to the fetus. Consumption of 930 (versus 480) mg choline/d increased circulating concentrations of choline derived methyl donors, restored the partitioning of choline between the CDP-choline and choline oxidative pathways to the nonpregnant state, and enhanced the use of choline as a methyl donor in both maternal and fetal compartments.

In conclusion, choline requirements are elevated in those with the *MTHFR* 677TT genotype and among third trimester pregnant women, and current recommendations may be suboptimal for these population sub-groups.

BIOGRAPHICAL SKETCH

Jian Yan grew up in Jinan, Shandong, China. He attended Shandong University and graduated in 2007 with a B.S. in Biology. During college, Jian worked in various research labs, including those specializing in protein crystallization, microbiology, plant physiology, and animal physiology. He found his passion for human nutrition during an internship at the Chinese Academy of Sciences, and started as a graduate student in the Division of Nutritional Sciences at Cornell University in the spring of 2008.

Jian started his lab rotation in Dr. Marie Caudill's lab in April of 2008, and has been working with Dr. Caudill on research projects investigating choline metabolism and choline dietary requirements. In 2011, his research was recognized by the Nutritional Sciences Council of the American Society for Nutrition, and he was awarded the Graduate Student Research Award. Jian was a teaching assistant from 2008 to 2011 for various undergraduate classes and was awarded the Outstanding Teaching Assistant award by the College of Agriculture and Life Sciences in 2011.

Dedicated to my parents and sister

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PREFACE

SPECIFIC AIMS

The overarching goal of my dissertation research was to quantify the effects of pregnancy, genetic variation, and choline intake on biomarkers of choline metabolism. In order to achieve this goal, two separate 12 wk feeding studies that employed stable isotope methodology were conducted.

Aim 1: To test the hypothesis that *MTHFR* C677T genotype and choline intake alter the metabolic use of orally consumed choline. This hypothesis was tested by evaluation of enrichments and enrichment ratios of choline derivatives in men with the *MTHFR* 677CC or 677TT genotype consuming ~ 15% of controlled choline intakes (550 or 1100 mg choline/d) as deuterium labeled *methyl*-d9-choline for the last 3-wk of a 12 wk controlled feeding study. *Results are presented in chapter 1.*

Aim 2: To test the hypothesis that pregnancy and choline intake alter markers of choline metabolism. This hypothesis was tested by evaluation of the blood and urinary concentrations of choline derivatives in nonpregnant and third trimester pregnant women consuming controlled choline intakes of either 480 (~ choline AI for pregnant women) or 930 mg choline/d for 12 wk. *Results are presented in chapter 2.*

Aim 3: To test the hypothesis that pregnancy and choline intake alter the metabolic use of orally consumed choline. This hypothesis was tested by evaluation of enrichments and enrichment ratios of choline derivatives in pregnant and nonpregnant women consuming ~ 22% of controlled choline intakes (480 or 930 mg choline/d) as deuterium labeled *methyl*-d9-choline during the last 6-wk of a 12 wk controlled feeding study. *Results are presented in chapter 3.*

This dissertation research yielded two published peer-reviewed articles (Chapters 1 & 2) and one original research manuscript, which will be submitted for publication within the next few months (Chapter 3).

BACKGROUND

Choline metabolism

Choline (2-hydroxyethyl-trimethyl-ammonium), a quaternary saturated amine, is an essential nutrient. Through its derivatives (**Figure 1**), choline plays indispensible roles in supplying methyl groups for one-carbon metabolism (via betaine) and maintaining biomembrane integrity (via phosphatidylcholine) [1].

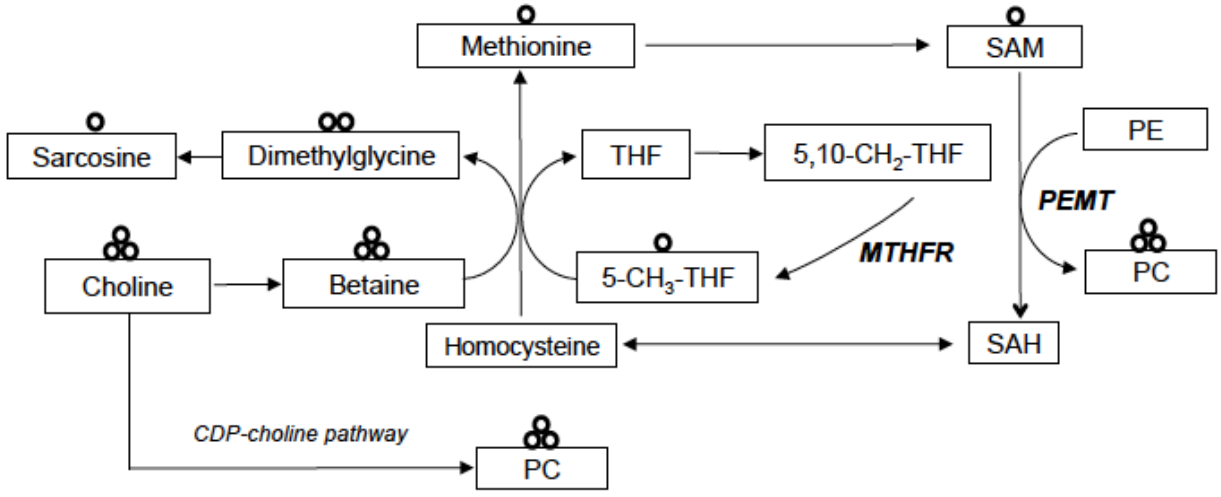

Figure 1 legend

Simplified diagram of choline related one-carbon metabolic pathways.

White circles indicate labile methyl groups associated with choline

Abbreviations: CDP-choline, cytidine diphosphate-choline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; 5-CH_3-THF, 5-methyl-tetrahydrofolate; 5,10-CH_2-THF, 5,10-methylene-tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; PEMT, phosphatidylethanolamine *N*-methyltransferase

Choline is a source of methyl groups for one-carbon metabolism

Choline can be oxidized to betaine, which donates a methyl group to homocysteine, producing methionine. The oxidation of choline also generates dimethylglycine (DMG) and methylglycine (sarcosine) in a sequential demethylation manner: choline → betaine → DMG → sarcosine. The methyl groups associated with these choline-derived methyl donors (i.e., DMG and sarcosine) can be used in folate mediated one-carbon (1-C) metabolism for the biosynthesis of nucleotides and methionine. Methionine serves as a precursor to *S*-adenosylmethionine (SAM), which is the major biological methyl donor for more than 60 methylation reactions, including DNA and histone methylation. Therefore, choline availability through influencing DNA/histone methylation has a downstream effect on genome stability and gene expression [1, 2].

There is an alternative homocysteine methylation step that does not utilize betaine. This pathway is dependent on the folate metabolite, 5-methyl-tetrahydrofolate (5-methylTHF). Thus, the metabolism of choline and the metabolism of folate intersect at the step of homocysteine methylation. The 5-methylTHF is produced from 5, 10-methyleneTHF by the enzyme 5, 10-methyleneTHF reductase (MTHFR). If the activity of MTHFR is modified, this may affect the availability of 5-methylTHF for methyl donation. Changes in folate derived methyl donor availability may subsequently affect the demand for betaine as a source of methyl groups for homocysteine remethylation [3].

A single nucleotide polymorphism (SNP) has been identified in the *MTHFR* gene (677C→T) [4]. Homozygotes for this SNP (*MTHFR* 677TT) have diminished enzyme activity versus *MTHFR* 677CC [4]. In the United States, the prevalence of the MTHFR 677TT genotype varies among different ethnic groups with an average of 10%. However the prevalence is 20% in people of Mexican descent [5]. MTHFR 677TT homozygosity is associated with an elevated

homocysteine level especially under conditions of folate insufficiency [6] and thus may increase the demand for choline derived methyl groups for homocysteine remethylation.

Choline and phosphatidylcholine inter-conversion

The choline derivative phosphatidylcholine may be produced via two pathways. The cytidine diphosphate-choline (CDP-choline) pathway utilizes the intact choline moiety to produce phosphatidylcholine. The CDP-choline pathway accounts for ~70% of total phosphatidylcholine production [7, 8]. Alternatively, phosphatidylcholine may be produced by three sequential methylations of phosphatidylethanolamine. This reaction is catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT) with three SAM molecules as the methyl donors. The PEMT enzyme is mainly expressed in the liver, and PEMT mediated phosphatidylcholine production accounts for ~30% of total phosphatidylcholine production [9].

Phosphatidylcholine is subject to hydrolysis by several phospholipases, which ultimately can convert phosphatidylcholine to free choline [10]. Hydrolysis of phosphatidylcholine from the CDP-choline pathway back to free choline provides no net gain of choline. However, hydrolysis of the PEMT derived phosphatidylcholine (which was initially produced from phosphatidylethanolamine that does not contain choline moiety) provides a net gain in free choline. Thus, PEMT mediated phosphatidylcholine synthesis is considered to be the denovo choline synthesis pathway and can supply in part the demand for choline.

Phosphatidylcholine is a major constituent of biomembranes. Choline requirements are thus high when a large quantify of phosphatidylcholine is needed to support rapid cell division and growth (e.g., during pregnancy) [11]. Phosphatidylcholine is also a major constituent of very-low-density lipoproteins (VLDL), which export lipids out of the liver [12]. Thus the most

obvious clinical symptom of choline deficiency is fatty liver, due to the lack of phosphatidylcholine for the synthesis and secretion of VLDL.

Choline adequate intakes (AI)

Despite the ability to endogenously produce choline via the PEMT pathway, studies have demonstrated that consuming a choline depletion diet resulted in liver dysfunctions [13]. This suggests that endogenous synthesis through the PEMT pathway alone is unable to meet choline demand. Therefore, the Food and Nutrition Board of the Institute of Medicine established dietary recommendations for choline for the first time in 1998 based on the amount needed to prevent liver dysfunction [14].

For adult men:

The choline AI for men is set at 7 mg/kg/day (~550mg/day) based on a single study conducted by Zeisel et al [13] reporting that a choline intake level of 500 mg/d (7 mg/kg/day) was adequate to prevent the choline deficiency induced liver malfunction evidenced by elevated serum alanine aminotransferase activity.

For adult women:

The AI for adult women was extrapolated from the AI for men, giving an AI of 425 mg/d.

For pregnant women:

Pregnancy is associated with a higher demand for choline due to accelerated one-carbon metabolism and the synthesis of new membranes as cells undergo division. IOM acknowledged this enhanced demand and set the AI for pregnant women (450 mg/d) as 25 mg/d higher than that of nonpregnant women (425 mg/d). The 25 mg/d incremental amount was the estimated choline demand to support fetal and placental growth and was largely based on animal data.

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CHAPTER 1

MTHFR C677T genotype influences the isotopic enrichment of one-carbon metabolites in folate-compromised men consuming d9-choline[*]

[*] Yan J, Wang W, Gregory JF, Malysheva O, Brenna JT, Stabler SP, Allen RH, Caudill MA. MTHFR C677T genotype influences the isotopic enrichment of one-carbon metabolites in folate-compromised men consuming d9-choline. Am J Clin Nutr 2011; 93:348-55.

ABSTRACT

Background: Homozygosity for the variant 677T allele in the methylenetetrahydrofolate reductase (MTHFR) gene increases the requirement for folate and may alter the metabolic use of choline. The choline adequate intake is 550 mg/d for men although the metabolic consequences of consuming extra choline are unclear.

Objective: Using deuterium labeled choline (d9-choline) as a tracer, the differential effects of the MTHFR C677T genotype and varied choline intake on the isotopic enrichment of choline derivatives were determined in folate-compromised men.

Design: Mexican American men with the MTHFR 677CC or 677TT genotype consumed a diet providing 300 mg choline/d plus supplemental choline chloride for total choline intakes of 550 (n=11; 4 677CC, 7 677TT) or 1100 mg/d (n=12; 4 677CC, 8 677TT) for 12-wk. During the last 3-wk, 15% of the total choline intake was provided as d9-choline.

Results: Low but measurable enrichments of the choline metabolites were achieved including d3-phosphatidylcholine (PtdCho), a metabolite produced in the endogenous pathway using choline derived methyl groups. Men with the MTHFR 677TT genotype had a higher urinary enrichment ratio of betaine to choline (P=0.041), a higher urinary enrichment of sarcosine (P=0.041), and on the 1100 mg/d choline intake level, a greater plasma enrichment ratio of d9-betaine to d9-PtdCho (P=0.033).

Conclusions: These data show for the first time in humans that choline itself is a source of methyl groups for denovo PtdCho biosynthesis and demonstrate that the MTHFR 677TT genotype favors the use of choline as a methyl donor.

INTRODUCTION

Choline is an essential micronutrient that serves as the precursor molecule for several important compounds including the phospholipids phosphatidylcholine (PtdCho) and sphingomyelin, the neurotransmitter acetylcholine, and the methyl donor betaine (1) (**Figure 1**). PtdCho and sphingomyelin are abundant in cellular membranes and have structural and signaling functions; PtdCho is also the major phospholipid in lipoproteins with influences on lipid metabolism and transport (2). Most PtdCho (~ 70%) is synthesized by the cytidine diphosphate (CDP)-choline pathway in which choline itself serves as the substrate; the remainder is synthesized *de novo* via phosphatidylethanolamine *N*-methyltransferase (PEMT), a S-adenosylmethionine (SAM)-dependent enzyme that sequentially methylates phosphatidylethanolamine to PtdCho (3). The methyl groups associated with SAM are originally derived from folate, choline (betaine) and/or methionine (4). In addition to PtdCho biosynthesis, these one-carbon units may be used for the biosynthesis of several metabolites, hormones, and neurotransmitters, and for the methylation of DNA with subsequent effects on gene expression and genome stability (5). Severe choline deficiency can cause metabolic disturbances such as fatty liver (6, 7), muscle damage (8), and aberrant gene expression as a result of alterations in DNA methylation patterns (9, 10). Associations between low dietary choline intake and increased risk for inflammation (11); birth defects (12); and breast cancer (13) also have been reported.

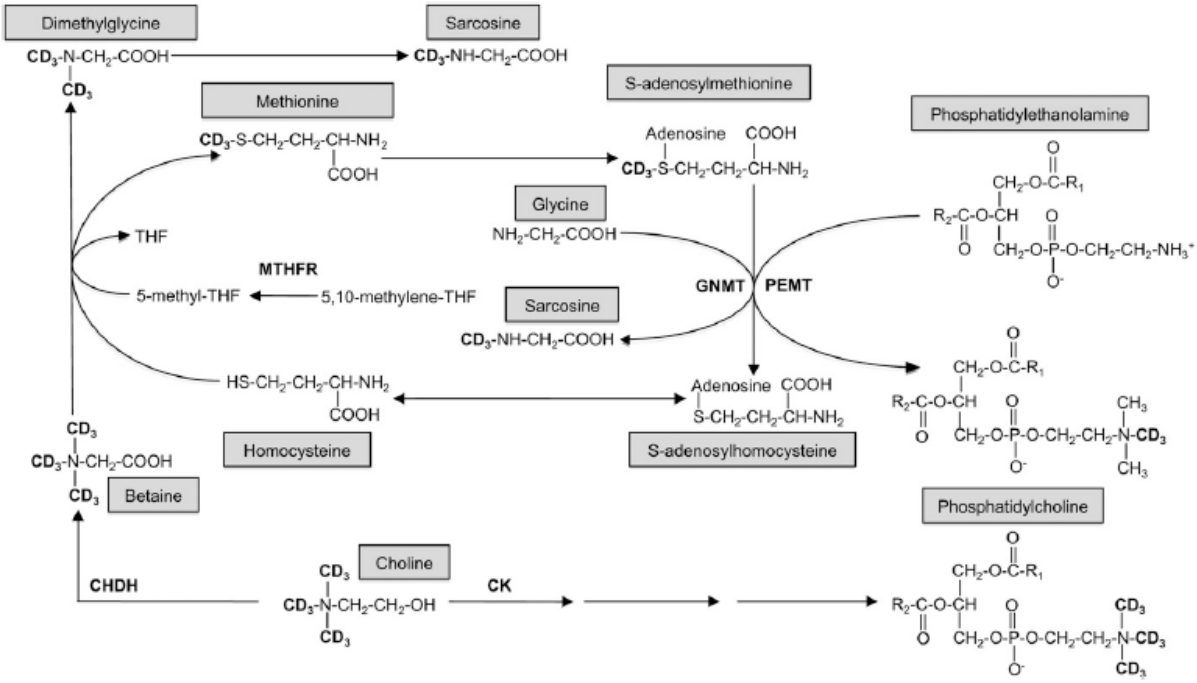

Figure 1 legend

The metabolic fate of the orally consumed deuterium labeled choline. The d9-choline tracer contained deuterium-labeled methyl groups facilitating the examination of the metabolic fate of choline-derived methyl groups in addition to the intact molecule. Abbreviations used: THF: tetrahydrofolate; MTHFR: 5,10-methylenetetrahydrofolate reductase; PEMT: phosphatidylethanolamine *N*-methyltransferase; GNMT: glycine *N*-methyltransferase; CK: choline kinase; CHDH: choline dehydrogenase

Based primarily on the amount of choline needed to prevent liver dysfunction, choline adequate intake (AI) levels of 425 and 550 mg/d for women and men respectively were established in 1998 (14). The metabolic requirement for choline is likely higher in individuals with compromised folate status because betaine (oxidized from choline) shares the homocysteine remethylation step in one carbon metabolism with 5-methyl-tetrahydrofolate (THF) (14). 5-methylTHF is derived from 5,10-methyleneTHF in a reaction catalyzed by 5,10-methyleneTHF reductase (MTHFR). The availability of 5-methylTHF, the main folate coenzyme in circulation, is modified by a common single nucleotide polymorphism, 677C→T, in the MTHFR gene (15).

We previously reported diminished serum folate and elevated plasma homocysteine concentrations in men with the MTHFR 677TT genotype, relative to those with the 677CC genotype, after 12 weeks of consuming the folate RDA (16). The diminished 5-methylTHF in men with MTHFR 677TT genotype may result in a higher reliance on betaine as the methyl group donor for one carbon metabolism and therefore alter choline partitioning/metabolism. Our finding of lower plasma phosphatidylcholine concentrations in this group of men with the MTHFR 677TT genotype (17) is consistent with this working hypothesis.

The objective of this study was to determine the effects of the MTHFR C677T genotype and/or varied choline intake on the metabolic use of orally consumed choline. To accomplish this aim, deuterium labeled choline (d9-choline) was consumed orally by men with the MTHFR 677CC or TT genotype during the last three weeks of a 12 week controlled feeding study. Isotopic enrichment and enrichment ratios (i.e., the proportion of the precursor molecule converted to the product molecule) of one carbon metabolites derived from the orally consumed labeled choline were evaluated.

SUBJECTS AND METHODS

Subjects and study design

The men in this study (n=23; 18 to 42y) represent a sub-sample of healthy Mexican American men (n=60; aged 18-55 years) pre-selected for the MTHFR 677CC or TT genotype and were recruited between June 2005 and September 2006. Additional inclusion criteria have been described elsewhere (16). The study was approved by the Institutional Review Board for Human Study Participants at Cal Poly Pomona University and written informed consent was given by each participant. Approval to use de-identified samples for the measurements made in this study was granted by the Cornell Institutional Review Board for Human Subjects.

This was a 12-week choline intervention study in which study participants (n=60) with the MTHFR 677CC (n=31) or 677TT (n=29) genotype were randomized at baseline to 300, 550, 1100 or 2200 mg/d choline. The diet, consumed by all study participants throughout the study, provided 300 mg/d total choline, 173 mg/d betaine as well as 319 μg/d natural food folate (16, 17). The study participants also consumed 70 μg supplemental folic acid / d for total folate intakes of 438 μg dietary folate equivalents / d (16). To achieve the target choline intakes of 550, 1100 or 2200 mg/d, unlabeled commercially available choline chloride (BCP Ingredients Inc, Verona, MO) was administered for the first 9 weeks as previously detailed (16). During the last three weeks of the study (wk 10-12), a sub-sample of the men in the 550 mg/d (n=11; 4 677CC, 7 677TT) and 1100 mg/d (n=12; 4 677CC, 8 677TT) choline intake groups received 15% of the target dose as d9-choline prepared from commercially available [trimethyl-d9]choline chloride (Cambridge Isotope Laboratories Inc, Andover, MA; **Figure 2**). In the 550 mg/d choline intake group, choline was derived from the diet (300 mg/d), unlabeled supplemental choline chloride (167.5 mg/d) and d9-labeled supplemental choline chloride (82.5 mg/d). The supplemental choline chloride (d0+d9) was

consumed daily at the breakfast meal. In the 1100 mg/d choline intake group, choline was derived from the diet (300 mg/d), unlabeled supplemental choline chloride (635 mg/d) and d9-labeled supplemental choline chloride (165 mg/d). The supplemental choline chloride (d0+d9) was divided into two equal portions (317.5 mg d0-choline plus 82.5 mg d9-choline) and consumed at breakfast and dinner.

The isotopic enrichment of choline and its metabolites was assessed at the end of the study (wk 12) in plasma and urine. Adherence to the study protocol was previously established by measurements of serum folate (a sensitive marker of dietary intake) and plasma free choline (16, 17).

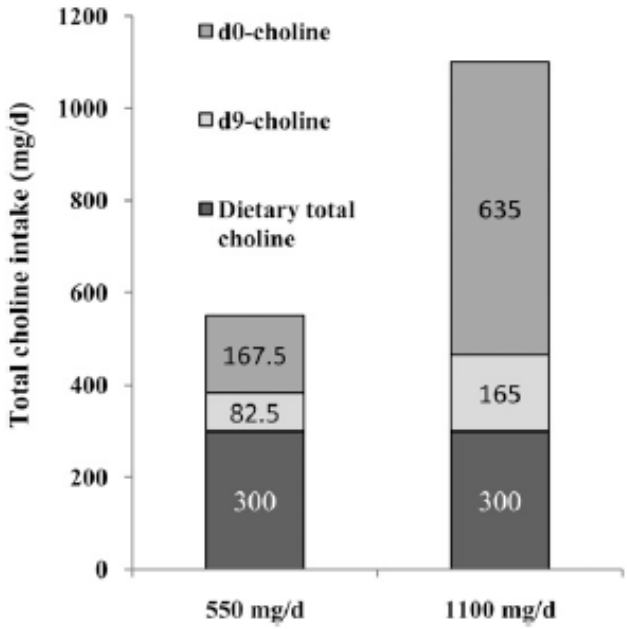

Figure 2 legend

Mean intake of dietary total choline and choline chloride either as unlabeled (d0) or labeled (d9) choline.

Sample collection and MTHFR C677T genotyping

Baseline and weekly fasting (10h) venous blood samples were collected into serum separator gel and clot activated tubes (Vacutainer; Becton Dickinson, Rutherford, NJ) and EDTA-coated tubes (Vacutainer), processed, and stored at -80^{o}C as previously described (18). Twenty-four hour urine collections were obtained at wk 0, 6, and 12, processed and stored at -20^{o}C as described previously (18). Determination of the MTHFR C677T involved polymerase chain reaction, digestion with Hinf1 and electrophoretic separation on an agarose gel (19).

Measurements of plasma and urinary choline metabolites

Plasma and urinary concentrations of free choline [unlabeled (d0) and d9]; betaine (d0 and d9); dimethylglycine (d0and d6); and sarcosine (d0 and d3) were determined by liquid chromatography tandem mass spectrometry (LC/MS/MS) according to the method of Holm et al (20). The system included an LCQ Advantage Mass Spectrometery system (Thermo Finnigan) with an electrospray ionization source operated in positive ion mode, a Surveyor HPLC system (Thermo Finnigan) and a refrigerated Surveyor autosampler (Thermo Finnigan). Urine or plasma (50 μL) was transferred to a 1.5 mL Eppendorf tube, and 100 μL of acetonitrile containing 0.1% (v:v) formic acid was added to precipitate proteins. d13-choline, d3-betaine, and d3-dimethylglycine (0.2 nmol/L of each) were used as internal standards. The mixture was vortexed and centrifuged at 4 ºC. An aliquot of supernatant (120 μL) was transferred to vials containing 120 μL 0.1% (v:v) formic acid in acetonitrile. The separation of choline, betaine and dimethylglycine was achieved using an Alltech® Prevail Silica analytical column (2.1×150 mm, 5 μm) and a Alltech® Prevail Silica guard column (2.1×7.5 mm, 5 μm) with a mobile phase of 81% filtered acetonitrile and 19% of 0.1% formic acid (v:v) in 15mM ammonium formate. The flow rate was 500μL/minute, the injection volume was 10 μL and the column temperature was maintained at 25 ºC.The external

standard curve for choline (d0 and d9), betaine (d0 and d9), and dimethylglycine (d0and d6) was constructed in 25:75 (v:v) 15mM ammonium formate and acetonitrile solution.

Plasma PtdCho (d0, d3, d6 and d9) was measured by LC/MS as described by Koc et al (21) with modifications based on our instrumentation (22) using d4-PtdCho as an internal standard. The calculation of enrichment percent (d3-Ptdcho/total Ptdcho; d9-Ptdcho/total Ptdcho) was based on the peak area under the curve.

Plasma methionine (d0, d3) was measured by gas chromatography-mass spectrometry (GC-MS) in negative ion electron capture mode as the n-propyl ester N-heptafluorobutyryl derivative using chemical ionization (23) as previously described (24).

A panel of urinary one-carbon metabolites (i.e., sarcosine, homocysteine, cystathionine, cysteine, methionine, aminobutyric acid, glycine and serine) was quantified by Gas Chromatography-Mass Spectrometry (GC-MS) following established protocol (25, 26). The enrichments of urinary DMG and sarcosine were also determined by GC-MS in a separate run in which internal standard was not added.

Statistical analysis

To test for differences in the dependent variables of interest (e.g., plasma d9-choline) between the MTHFR C677T genotypes and between two different choline intake groups, a 2-factor ANOVA (choline intake and MTHFR C677T genotype) was performed on each dependent variable. Dependent variables whose residuals were not normally distributed were log or square root transformed to fit the assumption of the ANOVA model. Effects were considered to be significant at $P < 0.05$ whereas a $P < 0.1$ was indicative of trends. Data are presented as means ± SEM for all dependent variables and were analyzed by SPSS software (version 15, SPSS Inc., Chicago, IL).

RESULTS

Characteristics of Study Population

Table 1 shows indicators of folate and choline status, as well as, concentrations of urinary metabolites related to one-carbon metabolism at the end of the 12 week study. Age and body mass index of the study population are also provided. Compared to men with the MTHFR 677CC genotype, those with the 677TT genotype had lower serum folate (P=0.003) and higher plasma homocysteine (P=0.001) concentrations. The mean age of men with the MTHFR 677TT genotype was also lower (P=0.038) relative to those with the 677CC genotype. Compared to men consuming 550 mg/d choline, the 1100 mg/d intake group had higher plasma betaine concentrations (P=0.014) and tended to have higher plasma free choline concentrations (P=0.052) and greater urinary cystathionine excretion (P=0.086). The MTHFR C677T genotype tended to interact with choline intake (P=0.057) to affect urinary homocysteine with the highest concentration detected in men with MTHFR 677TT genotype in the 1100 mg/d choline intake group. No other differences were detected.

TABLE 1

Clinical and biochemical variables at the end of the controlled feeding study (wk 12) in Mexican American men with the MTHFR 677CC or 677TT genotype consuming choline intakes of 550 or 1100[1].

	Choline intake, *mg/d*			***P*-values**		
Variable	550	1100	All	Genotype	Choline	Interaction
Age, y				0.038	0.927	0.604
677CC	28 (22-42)	26 (19-35)	27 (19-42)*			
677TT	21 (18-27)	22 (20-28)	22 (18-28)			
Total	24 (18-42)	24 (19-35)				
BMI, kg/m^2				0.652	0.317	0.179
677CC	24.5 (21-27)	28.8 (20-36)	26.6 (20-36)			
677TT	26.1 (22-30)	25.5 (21-30)	25.8 (21-30)			
Total	25.5 (21-30)	26.6 (20-36)				
Serum folate, nmol/L				0.003	0.676	0.915
677CC	12.3±1.7	11.6±2.7	11.9±1.5*			
677TT	7.3±0.9	6.9±0.8	7.1±0.6			
Total	9.1±1.1	8.5±1.2				
Plasma tHcy, μmol/L				0.001	0.328	0.336
677CC	11.7±0.7	11.8±0.7	11.7±0.5*			
677TT	28.5±6.7	40.7±5.7	35±4.5			
Total	22.4±4.9	31.1±5.6				
Plasma choline, μmol/L				0.655	0.052	0.841
677CC	7.0±0.8	9.4±2.2	8.2±1.2			
677TT	7.4±0.7	10.2±1.1	8.9±0.8			
Total	7.2±0.5	10.0±1.0$^{\#}$				

Plasma betaine, μmol/L				0.418	0.014	0.755
677CC	46.0±5.2	66.0±14.0	56±7.9			
677TT	42.6±3.1	58.3±4.7	51±3.5			
Total	43.8±2.6	60.9±5.4[#]				
Plasma total PtdCho (d0+d3+d9), μmol/L				0.498	0.733	0.079
677CC	1784±166	1532±93	1658±100			
677TT	1492±84	1665±101	1585±68			
Total	1599±88	1621±74				
Urinary choline, μmol/g Cr				0.135	0.085	0.249
677CC	16.9±6.3	22.7±5.5	19.4±4.1			
677TT	20.3±5.5	48.2±10	35.3±7.1			
Total	19.0±4.0	40.6±8.2				
Urinary betaine, μmol/g Cr				0.29	0.853	0.925
677CC	80.2±29	67.6±18	74.8±17			
677TT	124.6±55	120.5±31	122.4±29			
Total	106.9±34	104.7±23				
Urinary DMG, μmol/g Cr				0.159	0.957	0.088
677CC	86.6±23	35.7±2	69.6±18			
677TT	76.7±20	130.8±26	105.8±18			
Total	80.7±10	109.7±9				
Urinary sarcosine, μmol/g Cr				0.909	0.123	0.627
677CC	12.1±1.2	17.7±5.3	14.5±2.4			
677TT	13.1±2.5	16.1±1.7	14.7±1.5			
Total	12.7±1.6	16.5±1.7				
Urinary tHcy, μmol/g Cr				0.012	0.041	0.057
677CC	4.5±0.9	5.2±0.4	4.8±0.5			
677TT	7.7±3.4	25.8±9.1	18.9±5.2*			
Total	6.3±2.1	18.9±5.5[#]				
Urinary cystathionine, μmol/g Cr				0.787	0.086	0.859
677CC	4.9±1.2	7.2±1.2	6.0±0.9			
677TT	5.4±1.0	7.3±1.1	6.4±0.8			
Total	5.2±0.7	7.3±0.8[#]				

Urinary cysteine, mmol/g Cr				0.731	0.163	0.833
677CC	0.2±0.03	0.3±0.02	0.2±0.02			
677TT	0.2±0.02	0.2±0.02	0.2±0.01			
Total	0.2±0.01	0.2±0.01				
Urinary methionine, μmol/g Cr				0.356	0.135	0.673
677CC	6.3±1.7	8.6±1.9	7.4±1.2			
677TT	7.3±2.3	11.4±1.7	9.5±1.5			
Total	6.9±1.5	10.4±1.3				
Urinary aminobutyric acid, μmol/g Cr				0.944	0.517	0.713
677CC	5.7±1.1	5.9±1.4	5.8±0.8			
677TT	5.4±0.6	6.4±0.7	5.9±0.5			
Total	5.9±0.5	6.2±0.6				
Urinary glycine, mmol/g Cr				0.417	0.197	0.506
677CC	0.9±0.2	0.8±0.2	0.8±0.1			
677TT	1.1±0.2	0.8±0.1	0.9±0.1			
Total	1.0±0.1	0.8±0.1				
Urinary serine, mmol/g Cr				0.408	0.543	0.929
677CC	0.2±0.04	0.2±0.03	0.2±0.02			
677TT	0.3±0.04	0.2±0.04	0.2±0.03			
Total	0.2±0.03	0.2±0.03				

[1]Data are mean ± SEM or (range); *n* = 3-8 per group. BMI, body mass index; Cr, creatinine; DMG, dimethylglycine; PtdCho, phosphatidylcholine; tHcy, total homocysteine;

[#]Different from corresponding 550mg/d choline intake group, $P < 0.05$ (2-way ANOVA)

*Different from corresponding MTHFR 677CC genotype, $P < 0.05$ (2-way ANOVA).

Effects of choline intake and MTHFR C677T genotype on the isotopic enrichment and enrichment ratios of choline metabolites [Enrichment = labeled metabolite / (labeled + unlabeled metabolite]; [Enrichment ratio = enrichment of product / enrichment of precursor]

Label was detected at acceptable signal-to-noise ratios in most choline metabolites (**Figure 3**). Greater plasma enrichments of choline (*P*=0.029), betaine (*P* = 0.004), d3-PtdCho (*P* = 0.005) and d9-PtdCho (*P* = 0.009; Figure 3A) were observed in the 1100 versus the 550 mg/d choline intake group. Enrichments of plasma methionine (P=0.075), urinary choline (P=0.077) and urinary betaine (P=0.014) were also greater in the higher choline intake group (Figure 3A). However, choline intake did not affect the enrichment ratios of product:precursor metabolites (ie, betaine:choline, methionine:betaine, methionine:choline, d3-PtdCho:choline, d3-PtdCho:betaine) indicating that the enrichment of precursors and products were equivalently affected.

The MTHFR 677TT genotype yielded a lower enrichment of urinary choline (P=0.091) and a greater urinary betaine to choline enrichment ratio (P=0.041) which suggests that flux through choline dehydrogenase may be greater in men with the 677TT vs 677CC genotype (Figure 3B). In addition, the MTHFR C677T genotype and choline intake interacted to affect the plasma enrichment ratio of betaine to PtdCho derived from the CDP-choline pathway (Figure 3C). In men with the MTHFR 677TT genotype, the higher choline intake yielded a greater betaine to phosphatidylcholine enrichment ratio which suggests that betaine oxidation was favored over the use of choline by the CDP-choline pathway; no effect of choline intake on this ratio was observed in the 677CC genotype. No other significant effects of either choline intake, MTHFR C677T genotype or the interaction term were detected on the measured variables.

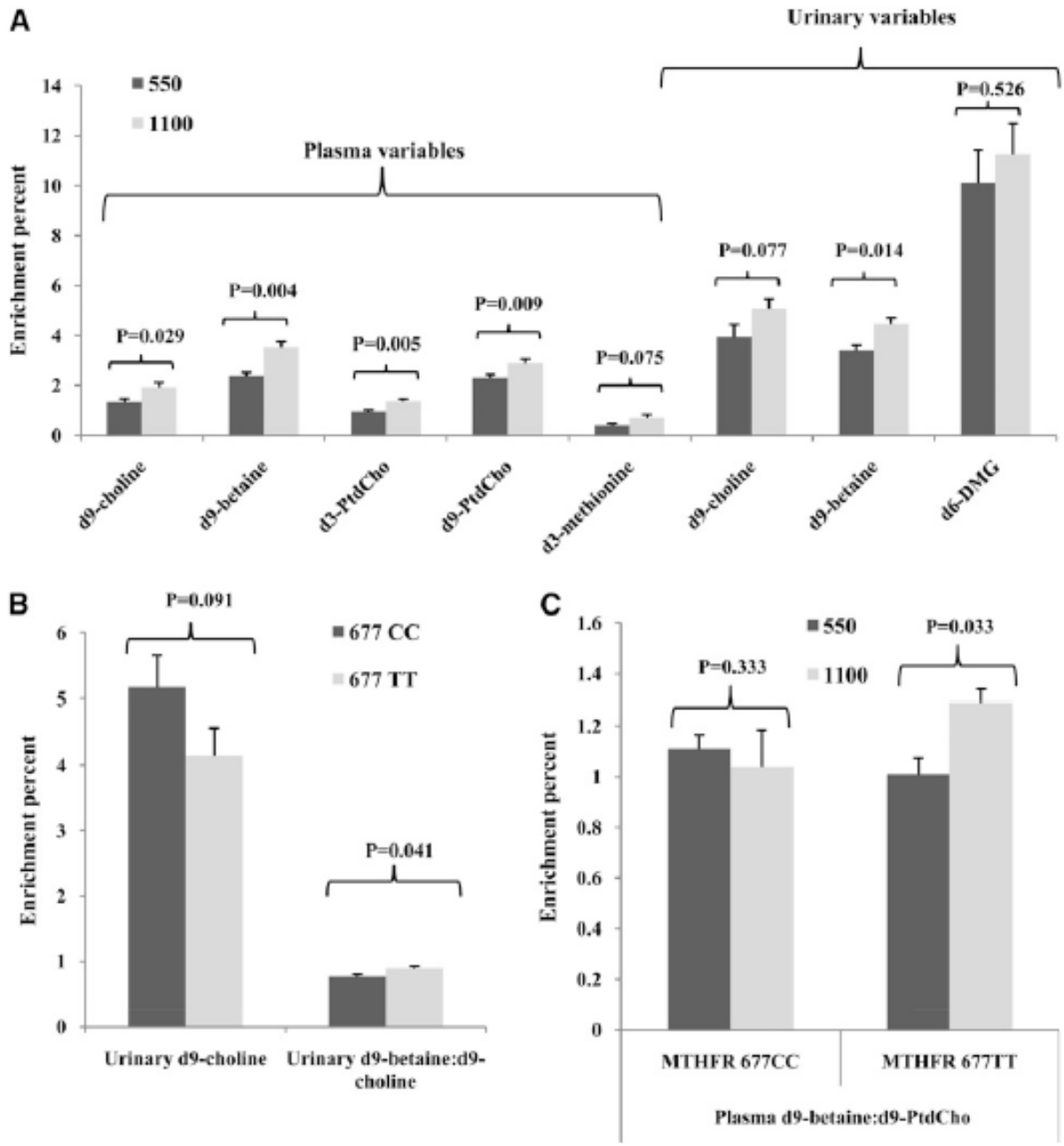
A
Urinary variables
550
1100
Plasma variables
Enrichment percent
P=0.526
P=0.077
P=0.014
P=0.004
P=0.009
P=0.029
P=0.005
P=0.075
d9-choline
d9-betaine
d3-PtdCho
d9-PtdCho
d3-methionine
d9-choline
d9-betaine
d6-DMG
B
P=0.091
677 CC
677 TT
Enrichment percent
P=0.041
Urinary d9-choline
Urinary d9-betaine:d9-choline
C
550
1100
P=0.333
P=0.033
Enrichment percent
MTHFR 677CC
MTHFR 677TT
Plasma d9-betaine:d9-PtdCho

Figure 3 legend

Isotopic enrichment of plasma and urinary choline metabolites [labeled metabolite / (labeled + unlabeled metabolite] grouped by choline intake (Panel A; n=10-12 per choline intake group), MTHFR C677T genotype (Panel B; n=7 677*CC*; n=13 677*TT*) or both (Panel C; n=4-8 per group) at week 12 in folate-compromised Mexican-American men consuming 15% of total choline intake as d9-choline from week 10-12. Data are presented as means ± SEM and were analyzed by a 2-factor ANOVA; $P < 0.05$ was considered statistically significant. For the enrichment ratio of d9-betaine to d9-PtdCho, an interaction between MTHFR C677T and choline intake was detected (P=0.03, Panel C). Thus the data are presented after stratifying by the MTHFR C677T genotype.

Effects of choline intake and MTHFR genotype on the proportion of PtdCho enrichment derived from the PEMT and CDP-choline pathways

Under the conditions of this study, only d3-PtdCho and d9-PtdCho (but not d6-PtdCho) were detected at quantifiable levels; therefore, it can be assumed that all of the d9-PtdCho was derived from the CPD-choline pathway. Notably, neither choline intake nor MTHFR C677T genotype affected the plasma enrichment ratio of d3-PtdCho to d9-PtdCho (data not shown). This observation suggests that the proportion of PtdCho derived from each of these pathways was unaffected by these variables.

Effects of choline intake and MTHFR genotype on urinary sarcosine enrichment

Urinary sarcosine enrichment was higher (P=0.049) in men with the MTHFR 677TT vs. 677CC genotype (**Figure 4**). Choline intake did not affect urinary sarcosine enrichment and the interaction term for MTHFR C677T genotype and choline intake was not significant.

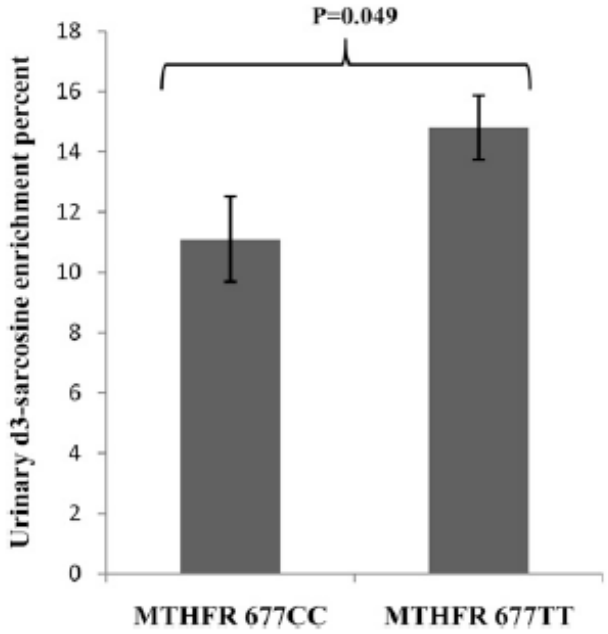

Figure 4 legend

Urinary sarcosine enrichment at week 12 according to MTHFR C677T genotype (n=7 677*CC*; n=13 677*TT*) in folate-compromised Mexican-American men consuming 15% of total choline intake (550 or 1100 mg/d) as d9-choline from week 10-12. Data are mean ± SEM and were analyzed by a 2-factor ANOVA; $P < 0.05$ was considered statistically significant. Choline intake did not affect urinary sarcosine enrichment and the interaction term for the MTHFR C677T genotype and choline intake was not significant

DISCUSSION

To the best of our knowledge, this is the first human study to employ stable isotope methodology to investigate the effects of choline intake and the MTHFR C677T genotype (677CC or 677TT) on the metabolic use of exogenous choline. The d9-choline tracer contained deuterium-labeled methyl groups and facilitated our examination of the metabolic fate of choline-derived methyl groups in addition to the intact molecule. In turn, distinctions could be made between PtdCho derived from the sequential methylation of phosphatidylethanolamine via PEMT which produces d3-PtdCho, d6-PtdCho and less likely d9-PtdCho versus PtdCho derived from intact choline by the CDP-pathway which yields only d9-PtdCho (Figure 1). Because d6-PtdCho was below the detection limits in the present study, it was assumed that all of the d9-PtdCho was derived from the CPD-choline pathway.

The d3 labeling of PtdCho demonstrates for the first time in humans that choline itself is a source of methyl groups for the denovo biosynthesis of the choline moiety through the PEMT pathway, a finding consistent with a cell culture model using rat primary hepatocytes (27). The observed d3 labeling of plasma methionine suggests that cellular pools of S-adenosylmethionine (SAM) were similarly labeled and, thus, served as methyl donors in d3-PtdCho synthesis by PEMT. Although the PEMT pathway is traditionally considered to be most important when dietary choline intake is low (28), d3 labeling of the PtdCho pool occurred under conditions of high choline intake (i.e., 1100 mg/d or two times the AI). Notably, the PtdCho molecules derived from the PEMT and CDP-choline pathways are molecularly distinct (29) with PEMT-derived PtdCho containing more polyunsaturated fatty acids including docosahexaenoic acid (DHA). Hence a possible caveat of increasing dietary intake of choline may be reducing the activity of the PEMT pathway which, in mice, results in decreased concentrations of circulating DHA (30). However, based on the enrichment

ratio of d3-PtdCho to d9-PtdCho, the relative proportions of PtdCho derived from each pathway were not altered by the choline intakes used in this study.

In the present study, we hypothesized that varied choline intake would affect the partitioning of choline such that a higher intake would favor its oxidation. However, the enrichment of products and precursors (i.e., d9-PtdCho/betaine; betaine/choline; DMG/choline) were affected to a similar extent suggesting that a doubling of choline intake did not alter the metabolic use of orally consumed choline (i.e., proportion of choline entering the oxidative pathway or the extent of its catabolism).

Simple one pool constant infusion kinetics requires that enrichments decrease as label moves from pool to pool. Our observed increase in isotope enrichment from precursor d9-choline to several products in blood and urine indicates that pools are in flux and that more than one precursor pool exists for derivation of subsequent products. For instance, we can speculate that the high enrichment of betaine, compared to choline, is due to: (i) a rise and fall of newly absorbed dietary choline as it is rapidly transported across the gut to the blood stream; and/or (ii) an increased release of more enriched products as a result of gut-level oxidation of choline to methylamines.

The observed greater enrichment of urinary metabolites than its plasma counterpart is likely due to the timing differences between the urine and blood collections. Similar to the kinetic curve in plasma, urinary metabolite enrichment is probably highest within several hours after dosing and declines thereafter reaching its lowest value the next day before dosing. Therefore the 24-h urine and fasting blood collections probably captured different phases of the kinetic curves with plasma enrichment reflecting the lowest point but urinary enrichment reflecting an average status.

MTHFR produces 5-methylTHF which, like betaine, can be used to remethylate homocysteine to methionine. The MTHFR 677TT genotype tends to yield diminished tissue concentrations of 5-methylTHF, particularly under conditions of folate inadequacy. Notably, in the present study, men with the MTHFR 677TT genotype had markedly diminished concentrations of serum folate (consisting mostly of 5-methylTHF; Table 1) such that 47% (7 of 15 participants) had serum folate concentrations in the deficient range (ie, $\leq$ 6.8 nmol/L; data not shown). Although, serum folate was also diminished in men with the MTHFR 677CC genotype (Table 1) only one participant had serum folate in the deficient range. As a result, the demand for betaine as a source of labile methyl groups for homocysteine remethylation may be higher in persons with the MTHFR 677TT genotype. The higher urinary enrichment ratio of betaine to choline in men with the 677TT genotype is consistent with this working hypothesis as it implies enhanced flux through choline dehydrogenase, the enzyme responsible for oxidizing choline to betaine. The higher plasma enrichment ratio of betaine to CDP-choline derived PtdCho in men with the MTHFR 677TT (relative to the 677CC genotype) on the higher choline intake is also consistent with an increased demand for betaine among MTHFR 677TT individuals as it suggests that more of the free choline was converted to betaine as opposed to entering the CDP-choline pathway. Increased demand for betaine in MTHFR deficiency has been reported in rodent studies as evidenced by diminished concentrations of hepatic phosphocholine and betaine concentrations in MTHFR +/- mice relative to +/+ mice (31).

The higher urinary enrichment of d3-sarcosine in men with the 677TT genotype is likely due to the faster oxidation of exogenous choline (choline → betaine → dimethylglycine → sarcosine) in these men and is consistent with their increased reliance on choline as a source of methyl groups. Sarcosine is also produced from glycine in a cytosolic reaction catalyzed by glycine *N*-methyltransferase (GNMT), an enzyme subject to reciprocal

regulation by SAM (positive cooperativity) and 5-methyltetrahydrofolate (allosteric inhibition) (32, 33). Given the lower serum folate in this group of men (Table 1), it is possible that a less inhibited GNMT led to greater production of sarcosine (and homocysteine) in those with the 677TT genotype.

Finally, these data show that daily oral administration of a choline tracer over a three week period leads to measurable enrichments in choline and its derivatives in humans. As such, these data can provide guidance for future studies designed to quantitatively assess the impact of varied choline intake or genetic variation on choline metabolism. The multiple methyl labeling strategy that we employed also suggests that inputs from SAM for PEMT-derived PtdCho can be estimated by conventional mass isotopomer distribution analysis (34), although somewhat higher labeling would be required to enable measurements of the d6 isotopomer which was below the detection limit in the present study. In principle, the mass spectra yield relative abundances of d0, d3, d6, and d9 choline isotopomers. The d3 and d6 concentrations represent the minimum of two isotopomers required to calculate enrichment of the precursor methyl pool. Analysis of that labeling would enable an estimate of the dilution and net flux of dietary choline-derived methyl groups as they pass through the PEMT pathway

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CHAPTER 2

Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans[*]

[*] Yan J, Jiang X, West AA, Perry CA, Malysheva OV, Devapatla S, Pressman E, Vermeylen F, Stabler SP, Allen RH, Caudill MA. Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. Am J Clin Nutr 2012; 95:1060-1071.

ABSTRACT

Background: In 1998, choline adequate intake (AI) levels of 425 and 450 mg/d were established for nonpregnant and pregnant women, respectively. However, no dose response studies have been conducted to evaluate the effects of pregnancy or maternal choline intake on biomarkers of choline metabolism.

Objective: We sought to quantify the effects of pregnancy and maternal choline intake on maternal and fetal indicators of choline metabolism.

Design: Healthy pregnant (n = 26, wk 27 gestation) and nonpregnant (n = 21) women were randomized to 480 or 930 mg choline/d for 12-wk. Fasting blood samples, placental tissue and umbilical cord venous blood were collected and analyzed for choline and its metabolites.

Results: Regardless of choline intake, pregnant women had higher ($P < 0.001$) circulating concentrations of choline (30%) but lower (13-55%; $P < 0.001$) concentrations of betaine, dimethylglycine, sarcosine and methionine. Obligatory losses of urinary choline and betaine were ~ 2-4 times greater ($P \leq 0.02$) among pregnant women. A higher choline intake yielded higher (12-46%; $P \leq 0.08$) concentrations of choline, betaine, dimethylglycine and sarcosine in both pregnant and nonpregnant women without affecting urinary choline excretion. The higher maternal choline intake also led to a doubling ($P = 0.002$) of dimethyglycine in cord plasma.

Conclusions: These data suggest that an increment of 25 mg choline/d to meet the demands of pregnancy is insufficient, and demonstrate that a higher maternal choline intake increases the use of choline as a methyl donor in both maternal and fetal compartments.

INTRODUCTION

Choline, a micronutrient commonly grouped with the B-vitamins, is required for the biosynthesis of phosphatidylcholine, the main component of cell membranes, and for the formation of the cholinergic neurotransmitter acetylcholine. Following its oxidation to betaine, choline is also a major dietary source of methyl groups, which can be transferred to DNA and histones in a S-adenosylmethionine (SAM) dependent reaction. DNA and histone methylation (i.e., epigenetic modifications) can in turn exert lasting effects on gene expression [1]. The oxidation of choline also generates dimethylglycine (DMG) and methylglycine (sarcosine) in a sequential demethylation manner: choline → betaine → DMG → sarcosine. The methyl groups associated with these choline-derived methyl donors (i.e., DMG and sarcosine) can be used in folate mediated one-carbon (1-C) metabolism for the biosynthesis of nucleotides and methionine [2].

Pregnancy is associated with a higher demand for choline due to accelerated 1-C metabolism and the formation of new membranes as cells undergo division [3]. Pregnancy causes a pronounced reduction of choline pools in rodents consuming a normal chow diet [4], indicating that the need for this nutrient by the mother and the fetus may exceed the amount consumed by the mother and the amount produced by the endogenous pathway (i.e., phosphatidylethanolamine *N*-methyltransferase pathway). Furthermore, supplementing the maternal rat diet with additional choline (approximately 4 times the amount in normal chow) leads to a significant enhancement of memory function of the adult offspring [5, 6] and substantially lessens aging-related memory decline [7, 8]. The long-lasting beneficial effects of maternal choline supplementation on cognitive function may arise, at least in part, from modifications of DNA methylation secondary to the use of choline as a methyl donor. Although few studies have addressed the adequacy of human choline intake during

pregnancy, dietary choline intake [9] and mid-gestation maternal serum total choline [10] are inversely associated with the risk of having a baby with a neural tube defect.

In 1998, dietary choline recommendations in the form of adequate intakes (AIs) were established for the first time by the Food and Nutrition Board of the Institute of Medicine [11]. The choline AI for pregnant women, 450 mg choline/d, is largely based on the AI for nonpregnant women (425 mg choline/d), which was extrapolated from a single study conducted in men and designed to prevent liver dysfunction [11]. An increment of 25 mg choline/d was added to the AI of nonpregant women to support fetal and placental growth and was largely based on animal data. Thus additional studies examining the effect of pregnancy and maternal choline intake on biomarkers of choline metabolism are needed to scientifically inform the development of dietary recommendations that optimize maternal health and fetal well-being.

The goals of this study were to quantify the effects of pregnancy on choline-derived methyl donors and related 1-C metabolites (e.g., homocysteine) under conditions of controlled intake and to determine whether maternal choline intake modulates these metabolic parameters in maternal, placental and fetal compartments.

STUDY PARTICIPANTS AND METHODS

Study Participants

Third trimester pregnant (gestational wk 27) and nonpregnant control women aged ≥21 y were recruited from Ithaca, NY between January 2009 and October 2010 by the use of approved flyers posted at local maternal clinics and at Cornell University. During the screening phase, all study volunteers completed a health history questionnaire which included (if applicable): participant's date of birth, gestational age at entry into prenatal care, date of last menstrual period, self-reported pre-pregnancy weight, gestational age at present, due date, health status, medication use, vitamin/mineral use, and drug/alcohol use. Entry into this study was contingent upon good health status as assessed by a blood chemistry profile, complete blood count and medical history questionnaire. Additional inclusion criteria included: (i) no tobacco or alcohol product use, (ii) no history of chronic disease, (iii) normal kidney and liver function, and (iv) willingness to comply with the study protocol (i.e., eating one meal per day at the on-site location and not consuming foods and beverages outside those provided by the study). Screening participants were excluded if they (i) were carrying more than one fetus, (ii) had pregnancy-associated complications (i.e., preeclampsia, gestational diabetes, or intrauterine growth restriction), or (iii) were taking prescription medications known to affect liver function.

Maternal and newborn information were obtained from medical charts. Maternal information included: total weight gained during pregnancy, due date, complications during pregnancy, and complications during labor. Newborn information included: date of delivery, mode of delivery, gestational age, length, weight, head circumference, and Apgar score.

The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and at Cayuga Medical Center (the

hospital where participants delivered their babies; Ithaca, NY). Informed consent was obtained from all participants prior to their entry into the study.

Study Design, Diet and Supplements

This is a 12-wk controlled feeding study in which healthy pregnant women (wk 27 gestation; n = 26) with singleton pregnancies and nonpregnant control women (n = 21) consumed 480 or 930 mg choline/day. The 7-day cyclical menu (**Table 1**) provided an average of 380 mg choline/day with 142 mg from water soluble forms (free choline, phosphocholine, and glycerophosphocholine) and 236 mg from lipid soluble forms (phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine). Supplemental choline chloride (Balchem, New Hampton, NY) was used to achieve the target intake levels and was consumed with a meal of the participant's choosing as a single bolus of 100 or 550 mg choline/d. For the period between completion of the 12-wk study and delivery of the baby, pregnant women who had not yet delivered (n = 23) continued to consume their assigned choline supplement (100 or 550 mg choline/d). Maternal blood (wk-0, 3, 6, 9, 10 and 12) and urine (wk-0, 6, 9 and 12) were collected throughout the feeding phase of the study; a maternal blood sample, placenta tissue and cord blood were obtained at delivery.

TABLE 1

Seven-day rotational menu consumed by pregnant and nonpregnant women throughout the 12-week study[1]

Day	Breakfast	Lunch	Dinner	Choline (mg)
Monday	2 Eggs (50g ea), scrambled 2 WW toast (28g ea) 1 Peach cup (133g) *Juice	Pesto Sandwich: 2 WW bread slices (28g ea) Pesto (15g) Swiss cheese (42g) Romaine lettuce (20g) Celery sticks (30g) Carrot sticks (30g)	Beef & Cheese Tacos: 3 Corn tortillas (33g ea) Ground beef (80% lean; 105g) Cheddar cheese (30g) Iceberg lettuce (20g) Melon (112g) Milk (284g)	Breakfast: 361 Lunch: 31 Dinner: 127 #Snack: 27 Total: 546 Water soluble: 117 Lipid soluble: 429
Tuesday	Waffle (130g) 1 Egg (50g), hard-boiled *Juice	Tuna Sandwich: 2 WW bread slices (28g ea) Tuna, canned (56g) Cheddar cheese (30g) Iceberg lettuce (30g) Mayonnaise (15g) Grapes (100g)	Spaghetti: Cooked pasta (220g) Tomato sauce (220g) Mushrooms (30g) Parmesan cheese (10g) Mozzarella cheese (25g) Milk (284g)	Breakfast: 189 Lunch: 57 Dinner: 107 #Snack: 27 Total: 380 Water soluble: 140 Lipid soluble: 240
Wednesday	Fitness crunch cereal (80g) Milk (284g) 1 Box of raisins (42g) 1 Medium banana (118g) *Juice	Pastrami Sandwich: 2 WW bread slices (28g ea) Pastrami (24g) Swiss cheese (23g) Romaine lettuce (30g) Cucumber (80g)	Vegetarian Pizza: Dough (200g) Tomato sauce (112g) Red peppers, jarred (60g) Mushrooms (30g) Spinach (20g) Mozzarella cheese (50g) Apple sauce (28g) Milk (284g)	Breakfast: 96 Lunch: 52 Dinner: 95 #Snack: 27 Total: 270 Water soluble: 152 Lipid soluble: 118

Thursday	2 Pancakes (100g ea) Blueberries, frozen (50g) *Juice	Egg Salad Sandwich: 2 WW bread slices (28g ea) 1 Egg (50g), hard-boiled Mayonnaise (20g) Romaine lettuce (45g) Celery sticks (30g) Carrot sticks (30g)	Beef & broccoli stir-fry: Beef (160g) Broccoli, frozen (110g) Rice (200g) Onions, frozen (13g) Melon (112g) Milk (284g)	Breakfast: 40 Lunch: 228 Dinner: 195 #Snack: 27 Total: 490 Water soluble: 167 Lipid soluble: 323
Friday	1 WW bagel (95g) 1 Orange cup (133g) *Juice	Bean Burrito: Black beans, canned (40g) Cheddar cheese (30g) Rice (30g) 1 Large WW tortilla (59g)	Lasagna: WW lasagna noodles, dry (23g ea) Tomato sauce (150g) Ground beef (80% lean; 60g) Cottage cheese (40g) Mozzarella cheese (10g) Parmesan cheese (10g) Summer squash (40g) Zucchini (40g) Milk (284g)	Breakfast: 50 Lunch: 34 Dinner: 168 #Snack: 27 Total: 279 Water soluble: 137 Lipid soluble: 142
Saturday	2 Raspberry muffins (60g ea) 1 Medium banana (118g) *Juice	Turkey Sandwich: 2 WW bread slices (28g ea) Turkey (25g) Provolone cheese (23g) Iceberg lettuce (20g) Cucumber (80g)	Chicken Quesadilla: Chicken (65g) 2 Large WW tortillas (59g ea) Cheddar cheese (150g) Corn, frozen (100g) Milk (284g)	Breakfast: 122 Lunch: 38 Dinner: 178 #Snack: 27 Total: 365 Water soluble: 137 Lipid soluble: 228

Sunday	Oat Granola cereal (80g) Milk (284g) 1 Box of raisins (42g) 1 Peach cup (133g) *Juice	Vegetable soup, (396 g) Corn muffin (60g) Grapes (100g)	<u>Goulash:</u> Cooked pasta (200g) Beef (120g) Onion, frozen (30g) Red peppers, jarred (30g) Tomatoes, canned (50g) Tomato puree (100g) Potatoes, canned (35g) Pineapple, canned (70g) Milk (284g)	Breakfast: 64 Lunch: 59 Dinner: 170 #Snack: 27 Total: 320 Water soluble: 149 Lipid soluble: 171

[1]Dietary derived choline includes water-soluble forms (free choline, phosphocholine and glycerophosphocholine) and lipid-soluble forms (phosphatidylcholine, sphingomyelin and lysophosphatidylcholine)

*Juice choices included apple, crangrape or cranberry

#Snacks were provided daily and included: 170 g yogurt (vanilla, raspberry or peach flavored) and 156 g V8 juice.

Abbreviations used: WW, Whole Wheat

All meals and snacks were prepared in the Human Metabolic Research Unit at Cornell University. Study participants were required to consume at least one meal per day in the metabolic unit Monday through Friday; all other meals/snacks and beverages were provided as takeaways. The basic menu provided 2100 kcal/d. Energy intake was modified without affecting choline consumption by the addition or subtraction of the following food items: unenriched white rice, snack foods (i.e., chips, popcorn, rice cakes and apples) and certain beverages (i.e., soda, lemonade and apple juice). Weight was obtained weekly to monitor weight gain in the pregnant women and weight maintenance in the nonpregnant women. The investigators had daily contact with the participants throughout the study which enhanced their compliance to the dietary regimen. In addition, the participants were required to verify consumption of each food item by completing a daily check list provided by the investigators.

The choline stock solution was prepared every 3-mo by dissolving choline chloride in autoclaved drinking water, filtering the solution through 0.22 μm filters, dispensing it into amber glass bottles, and storing it at 4°C. The molarity of the choline stock solution was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The variability between the choline stock solutions throughout the feeding trials was < 5%. Periodic testing of the choline stock solution during the 3-mo period indicated no degradation. Appropriate volumes of the choline stock solution were dispensed into 50-mL conical tubes, mixed with crangrape juice, and stored at -20°C. The choline crangrape cocktail was thawed 1–2 d prior to consumption and kept at 4°C. During the last 6-wk of the study (wk 6 - 12), 20% of the total choline intake was provided as deuterated methyl-d9-labeled choline (d9-choline; Cambridge Isotope Laboratories, Andover, MA). The administration of labeled choline will allow for future examinations of the activity of the phosphatidylethanolamine *N*-methyltransferase pathway through isotopomer

analysis. The d9-choline stock solution was prepared the same way as the unlabeled choline stock solution and stored at 4°C in amber glass bottles. Appropriate volumes of the d9-choline were dispensed into 50 mL conical tubes, mixed with crangrape juice, and stored at -20°C until used for consumption.

A prenatal multivitamin (Pregnancy Plus, Fairhaven Health, LLC, Bellingham, WA) and docosahexaenoic acid (200 mg; Neuromins, Nature's Way Products, Springville, Utah) were provided daily and a potassium and magnesium supplement (General Nutrition Corp, Pittsburgh, PA) was provided three times per week to all study participants. Throughout the week, all supplements (including choline) were consumed at the on-site meal of the participant's choosing (i.e., breakfast, lunch or dinner) under the supervision of the investigators. For the weekend, the participants were asked to consume the supplements with a meal of their choosing and to return all containers/disposables on Monday.

Overall, the study protocol was well-tolerated with 92% of the participants completing the study regimen (21 of 22 nonpregnant women, 26 of 29 pregnant women). Reasons for stopping the study included nausea (n = 1), early delivery (n = 1), personal challenges (n = 1), and food dislikes (n = 1). Additional information regarding the flow of study participants through the screening and intervention phases is shown in **Figure 1**.

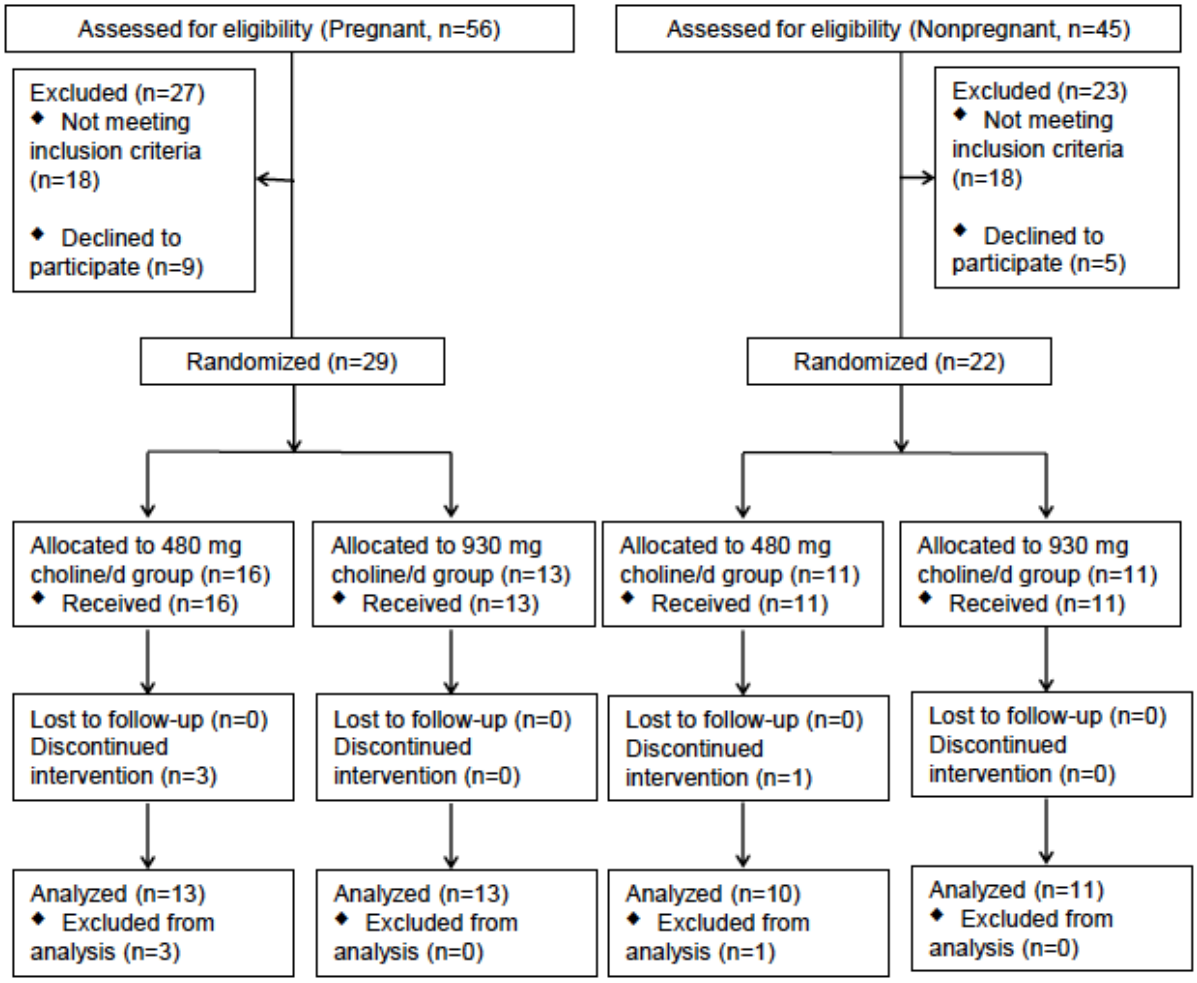

Figure 1 legend

Flow of participants throughout the study screening and intervention phases.

Sample collection and processing

Fasting blood samples (10-h) at wk-0, 3, 6, 9, 10 and 12 were collected in one 10 mL serum separator gel and clot-activator tube (SST Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ); three 10 mL EDTA tubes (Becton, Dickinson and Company); and one 5 mL EDTA tube (Becton, Dickinson and Company). The three 10 mL tubes containing EDTA blood were put on ice immediately following the blood collection and centrifuged within 90-min at 1055 x *g* for 15-min at 4°C. Buffy coat, plasma and whole blood were removed for genotyping and metabolite measurements as previously described [12, 13]. The SST-blood was kept at room temperature, allowed to clot and centrifuged at 650 x *g* for 15-min at room temperature. All of the biological samples were dispensed into 1.8 mL cryostat vials (CryoTube; NUNC, Roskilde, Denmark) and stored at -80°C.

Maternal blood and cord blood samples were also collected. Maternal blood samples were collected into one 10 mL EDTA tube and one 10 mL SST tube at the hospital within 24-h prior to delivery. The cord blood samples were collected into one 10 mL EDTA tube at the time of delivery. After collection, the maternal and cord blood samples were placed on ice (or refrigerated at 4°C) and processed as described previously within 4-h.

The placenta was obtained at delivery and processed at the hospital within 90-min of delivery. After removal of the amnion, twenty full thickness tissue biopsies (~ 0.5 cm x 0.5 cm x 0.5 cm) were taken from four separate locations (i.e., the placenta was visually divided into four quadrants). Samples were then rinsed with PBS immediately. One sample from each quadrant was randomly selected for RNA extraction and RNA stabilization solution (Qiagen, Valencia, CA) was added to prevent RNA degradation. The other samples were flash frozen in liquid nitrogen, placed in a cryostat tube and stored temporarily in a canister containing liquid nitrogen.

In the lab, all samples were stored at -80°C until subsequent analyses. Anthropometric measurements of the whole placenta were obtained and included length, width, depth and weight; weight and length of the umbilical cord were also recorded.

In addition, 24-h urine samples were collected at wk-0, 6, 9 and 12 in amber wide mouth polyethylene bottles (1 L, Nalgene, Rochester, NY) containing 5 g of sodium ascorbate. Participants were provided with an insulated bag with ice packs and instructions to keep the urine cold at all times. For each collection, the urine was pooled, total volume recorded, and urine was dispensed into five 125 mL narrow-mouth field sample bottles (ThermoFisher, Waltham, MA). Aliquots of 1 mL were placed into cryostat vials. All samples were stored at -80°C.

Analytical Measurements

Choline content of diet

The choline content of the diet was determined before starting the study and once during the study. Each meal (i.e., breakfast, lunch, dinner, or snack), including beverage, was prepared as for the participant's consumption, and blended with 150 mL of cold 0.1 mol potassium phosphate buffer/L (pH = 6.3) that contained 57 mmol ascorbic acid/L. This mixture was dispensed into 50 mL conical tubes and stored at -80°C. Total choline and betaine contents were determined by the method developed by Koc et al. [14] with modifications based on our instrumentation consisting of a TSQ Quantum mass spectrometer with electrospray ionization source (Thermo, San Jose, CA) equipped with a refrigerated Accela autosampler (Thermo) and an Accela pump with degasser (Thermo). Extraction and quantification procedures were detailed previously [15]. The average choline and betaine contents of the diet were 380 and 100 mg/d, respectively. Lipid-soluble forms of choline (i.e., phosphatidylcholine, lysophosphatidylcholine

and sphingomyelin) accounted for ~ 2/3 of dietary choline content and the remaining 1/3 was derived from water-soluble forms of choline (i.e., free choline, phosphocholine and glycerophosphocholine) (Table 1).

Plasma, Serum, Urine and Tissue Measurements

LC-MS/MS was used to measure plasma, urinary, and placental concentrations of free choline, betaine, DMG and/or acetylcholine [16, 17] with modifications based on our instrumentation [18]. Serum and urinary homocysteine, sarcosine and methionine were quantified by GC-MS using established protocols [19, 20] .

As recent findings indicate that the gut microbiome influences choline metabolism and, in turn, the risk of cardiovascular disease [21], blood and urinary trimethylamine *N*-oxide (TMAO), a gut-flora-dependent choline oxidative metabolite, were also measured by the method of Wang et al. [21] with modifications based on our LC-MS/MS instrumentation. Briefly, urine (5 μL) or plasma (50 μL) were spiked with $^{13}C_3$-TMAO as an internal standard and mixed with acetonitrile containing 0.1% formic acid to precipitate protein. After vortexing, the samples were centrifuged (10,600 x *g* for 5-min at 4°C) and the supernatant was transferred to a vial from which 10 μL was injected into the LC-MS/MS system. Metabolites were separated by HPLC using a Prevail silica column (150 × 2.1 mm, 5 μm; Grace, Deerfield, IL) with matching guard column (4.6 × 25 mm, 5 um). The mobile phase was run under isocratic conditions (500 μL/min) and contained acetonitrile (81%) and ammonium formate (15 mM) with 0.1% formic acid. Analyses were performed using electrospray ionization in positive-ion mode with multiple reaction monitoring of the following transitions: m/z 76.3 > 58.4 for TMAO; m/z 79.2 > 61.4 for $^{13}C_3$-TMAO; and m/z 85.3 > 66.4 for TMAO-d9. Internal standard, $^{13}C_3$-TMAO, was synthesized in house from $^{13}C_3$-TMA hydrochloride (SIGMA, St. Louis, MO) using hydrogen

peroxide [22]. Briefly, 30% hydrogen peroxide and 33% of $^{13}C_3$-TMA hydrochloride solution were mixed (vol:vol = 6:1) and left at room temperature overnight. The mixture was then dried, re-suspended in water and tested with LC-MS/MS. Varying levels of TMAO diluted with water were used to produce standard curves for urinary TMAO quantification. The curve was generated by plotting the peak area ratios versus the concentrations. Standard curves of plasma TMAO were constructed by spiking varying levels of TMAO into control plasma. Spiked TMAO area ratios (measured TMAO area ratios minus endogenous TMAO area ratio) were plotted against the spiked concentrations to generate the standard curves.

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides were measured on automated chemistry analyzer Dimension Xpand Plus (Siemens Healthcare Diagnostics, Deerfield, IL) at the Human Metabolic Research Unit Clinical Chemistry Lab at Cornell University; serum and red blood cell folate were measured microbiologically as previous described [13].

Choline acetyltransferase (ChAT) transcript abundance

Quantitative real-time RT-PCR was carried out to analyze the expression of *ChAT* in placental tissues. The RNA extraction, reverse transcription and real-time PCR procedures were as previously described [23]. Forward and reverse primers for *ChAT* were: 5'-TGGACATGATTGAGCGCTGC-3' and 5' –CGTTCTTGCTGTAGCCTCCG-3', respectively. Data are expressed as fold-change of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase *(GAPDH).*

Statistical Analysis

Forty-seven women (21 nonpregnant, 26 pregnant) finished the study and were included in the statistical analysis. With the exception of three pregnant women at wk-12, data were available for every time point (wk-0, 3, 6, 9, 10 and 12 for blood; wk-0, 6, 9 and 12 for urine). For wk-12, three pregnant women (1 in 480 mg choline/d group; 2 in 930 mg choline/d group) delivered during the final week of the study. For these participants, the maternal blood sample obtained at delivery was used.

Plots and histograms of the residuals were used to assess normality and variance homogeneity. Data that deviated from the normal distribution (i.e., plasma betaine, plasma DMG, serum sarcosine, urinary choline, urinary betaine, urinary DMG, urinary sarcosine and urinary homocysteine) were logarithmically transformed to achieve normality and the transformed data were used in subsequent analyses.

To test for baseline (wk-0) differences in the dependent variables between pregnant, nonpregnant women and choline intake groups, a two-way ANOVA (choline intake and pregnancy) was performed.

To delineate the impact of pregnancy on the dependent variables through time, a mixed linear model was constructed for each dependent variable. As pregnancy is a physiologic state, not a treatment, baseline (wk-0) measures of the dependent variables were included as a data point. Pregnancy, continuous time (e.g., wk-0, 3, 6, 9, 10 and 12 for plasma variables) and their interaction were included as fixed effects; sample ID was included as a random effect. In the initial analysis, choline intake and its interactions with pregnancy and continuous time were also included as fixed effects; however, no three-way (choline intake x pregnancy x time) or two-way

(choline intake x pregnancy) interactions were detected. Thus, choline intake was not included in the final model.

To delineate the impact of choline intake on the dependent variables, a mixed linear model was constructed for each dependent variable after grouping by pregnancy state. As baseline (wk-0) measures may influence the response to choline treatment, measures of the dependent variables at wk-0 were included as a covariate. Choline intake, continuous time (e.g., wk-3, 6, 9, 10 and 12 for plasma variables) and their interaction were included as fixed effects; sample ID was included as a random effect; wk-0 measure was included as a covariate.

Candidates for entry as covariates into both GLMs were age, BMI, and serum folate at wk-0. The covariates not achieving a statistical significance of $P \leq 0.05$ were removed from the models in a stepwise manner.

To test for differences in the plasma and urinary TMAO, a two-way ANOVA (choline intake and pregnancy) was performed with the change through time (wk-12 *minus* wk-0) as the dependent variable and choline intake, pregnancy as well as the baseline (wk-0) measure as the independent variables. To test for differences in the placental and fetal dependent variables, a one-way ANOVA (choline intake) was performed. Paired t-tests were also performed to compare maternal (at delivery) and fetal corresponding variables.

The data were analyzed using SPSS software (version 19; SPSS Inc, Chicago, IL). Differences were considered to be significant at $P \leq 0.05$. Data are presented as means ± SD for the dependent variables (unless noted otherwise).

RESULTS

Subject characteristics and baseline (wk-0) measures

Twenty-six third trimester pregnant women and 21 nonpregnant women successfully completed the feeding study. The characteristics and baseline (wk-0) measures of the final study group stratified by pregnancy and choline intake group assignment are shown in **Table 2**. The ethnic distribution across the choline intake groups was balanced and reflected the region from which the women were recruited. The self-report prepregnancy BMI of pregnant women did not differ from nonpregnant women ($P = 0.9$). The weights of nonpregnant women were maintained within ± 8% of initial values. For pregnant women, the average weight gain from wk-0 to wk-12 of the study was 6.3 ± 2.3 kg (13.9 ± 5.1 lb) and did not differ ($P = 0.8$) between choline intake groups.

Indicators of liver function (serum ALT and AST) and kidney function (serum BUN) were within normal ranges at baseline (Table 1) and throughout the study for all participants (data not shown). Serum total cholesterol, LDL-C, HDL-C and triglycerides were 1.3-2.4 times higher ($P < 0.001$) among third trimester pregnant versus nonpregnant women. Serum folate and RBC folate were well above levels associated with deficiency (> 3 ng/mL for serum folate and > 140 ng/mL for RBC folate) with greater concentrations among pregnant women ($P < 0.01$) due to consumption of a prenatal vitamin containing folic acid by 85% of the pregnant women before the onset of the study.

At baseline, plasma free choline tended to be higher (19%; $P = 0.09$) while plasma betaine, plasma DMG, serum sarcosine, serum homocysteine and serum methionine were 57%, 44%, 45%, 43% and 15% lower ($P < 0.05$) respectively among third trimester pregnant versus nonpregnant women. Urinary free choline excretion was higher ($P < 0.001$) among pregnant

women and was closely associated (Pearson's coefficient $r = 0.6$; $P = 0.004$) with plasma total cholesterol. Plasma TMAO did not differ between pregnant and nonpregnant women ($P = 0.5$) but was lower ($P = 0.025$) in the women assigned to the high choline intake group. No differences ($P > 0.2$) in urinary TMAO were detected between assigned choline intake groups and/or between pregnant and nonpregnant women.

TABLE 2

Baseline (wk-0) measures of clinical and biochemical variables in nonpregnant and third trimester pregnant women randomized to 480 or 930 mg choline/d[1,2]

	Nonpregnant women, *n* = 21			Pregnant women, *n* = 26		
Variables	480 mg/d	930 mg/d	All	480 mg/d	930 mg/d	All
Age (y)	29 (21 - 37)	29 (23 - 40)	29 (21 - 40)	29 (25 - 33)	28 (22 - 34)	28 (22 - 34)
Ethnicity						
African American (N)	1	1	2	0	1	1
Asian (N)	0	1	1	1	2	3
Caucasian (N)	7	6	13	9	7	16
Latino (N)	2	2	4	2	2	4
Other (N)	1	0	1	1	1	2
Gestational age (week)	-------	-------	-------	27 (26-29)	27 (26-28)	27 (26-29)
Prepregnant BMI (kg/m^2)	23.7 (19.6-27.3)	23.5 (18.2-29.8)	23.6 (19.6-29.8)	23.6 (20.2-31.9)	23.7 (19.9-29.8)	23.6 (19.9-31.9)
Serum ALT (U/L)	33 ± 8	30 ± 5.5	31 ± 6.8	36 ± 14	30 ± 7.2	33 ± 11
Serum AST (U/L)	18 ± 6	17 ± 4	18 ± 5	21 ± 7	18 ± 5	20 ± 6
Serum BUN (mg/dL)	10 ± 3	11 ± 4	11 ± 3	7 ± 2	7 ± 2	7 ± 2 [c]
Serum cholesterol (mg/dL)	169 ± 34	160 ± 34	164 ± 33	259 ± 31	238 ± 33	249 ± 33 [c]
Serum HDL-C (mg/dL)	57 ± 11	61 ± 13	59 ± 12	77 ± 22	75 ± 11	76 ± 17 [c]
Serum LDL-C (mg/dL)	98 ± 30	91 ± 18	95 ± 23	149 ± 24	142 ± 35	146 ± 29 [c]
Serum triglycerides (mg/dL)	81 ± 29	55 ± 18 [*]	68 ± 27	176 ± 55	139 ± 34 [*]	158 ± 49 [c]
Plasma choline (μmol/L)	6.2 ± 1.6	6.1 ± 1.6	6.2 ± 1.6	6.5 ± 1.1	7.4 ± 2.0	7.0 ± 1.7
Plasma betaine (μmol/L)	22 ± 10	28 ± 10	25 ± 10	11 ± 3	12 ± 5	11 ± 4 [c]
Plasma DMG (μmol/L)	2.5 ± 2.3	2.2 ± 1.2	2.3 ± 1.8	1.2 ± 0.5	1.5 ± 0.6	1.3 ± 0.6 [a]
Serum sarcosine (μmol/L)	1.0 ± 0.3	1.2 ± 0.5	1.1 ± 0.4	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.2 [c]
Serum Hcy (μmol/L)	7.0 ± 2.0	6.4 ± 0.8	6.7 ± 1.5	4.2 ± 1.0	3.5 ± 0.6	3.8 ± 0.9 [c]
Serum methionine (μmol/L)	26 ± 3.9	26 ± 3.1	26 ± 3.4	22 ± 1.8	22 ± 1.8	22 ± 1.8 [c]

Plasma TMAO (μmol/L)	2.5 (0.3 - 10.8)	1.2 (0.4 - 5.6) *	1.8 (0.3 - 10.8)	2.5 (0.5 - 15.7)	1.1(0.2 - 5.8) *	1.8(0.2 - 15.7)
Serum folate (ng/mL)	20 ± 8	18 ± 7	19 ± 7	29 ± 10	30 ± 18	30 ± 14 [b]
RBC folate (ng/mL)	472 ± 137	560 ± 139	518 ± 142	920 ± 190	920 ± 387	920 ± 299 [c]
Urinary choline (mg/g cr)	1.9 ± 0.7	2.0 ± 0.9	2.0 ± 0.8	9.2 ± 6.4	9.2 ± 5.9	9.2 ± 6.0 [c]
Urinary betaine (mg/g cr)	8.6 ± 5.7	6.4 ± 2.5	7.5 ± 4.1	15.5 ± 9.6	10.6 ± 3.9	13.1 ± 7.6 [b]
Urinary DMG (mg/g cr)	3.2 ± 2.0	3.1 ± 1.5	3.2 ± 1.7	3.5 ± 2.4	4.4 ± 1.8	4.0 ± 2.1
Urinary sarcosine (mg/g cr)	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.4	0.5 ± 0.3 [b]
Urinary Hcy (mg/g cr)	0.5 ± 0.3	0.5 ± 0.2	0.5 ± 0.3	0.6 ± 0.3	0.7 ± 0.4	0.7 ± 0.3
Urinary methionine(mg/g cr)	1.6 ± 0.6	1.2 ± 0.4	1.3 ± 0.6	2.7 ± 1.3	3.0 ± 2.0	2.8 ± 1.7 [c]
Urinary TMAO (mg/g cr)	9.9 (3.6 - 18.7)	11 (2.3 - 23)	10.5 (2.3 - 23)	12 (1.3 - 47)	7.4 (1.7 - 26)	9.6 (1.3 - 47)

[1]Data are presented as mean ± SD or (range).

[2]Data were analyzed by 2-way ANOVA; n = 10~13 per group. No significant interactions ($P > 0.1$) between pregnancy and choline intake were detected at baseline for any of the dependent variables.

Pregnant versus nonpregnant women: [a] indicates $P \leq 0.05$; [b] indicates $P \leq 0.01$; and [c] indicates $P \leq 0.001$

480 versus 930 mg choline/d group assignment: * indicates $P \leq 0.05$

Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; DMG, dimethylglycine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RBC, red blood cell; Hcy, total homocysteine; TMAO, trimethylamine-*N*-oxide.

Effects of pregnancy on blood and urinary biomarkers of choline metabolism

Main effects of pregnancy

Throughout the study, circulating concentrations of choline metabolic biomarkers differed between third trimester pregnant and nonpregnant women. On average, third trimester pregnant women had 30% higher ($P < 0.001$) plasma free choline, 55% lower ($P < 0.001$) plasma betaine, 38% lower ($P < 0.001$) plasma DMG, 49% lower ($P < 0.001$) serum sarcosine, 33% lower ($P < 0.001$) serum homocysteine, and 13% lower ($P < 0.001$) serum methionine than nonpregnant women (**Table 3)**.

Urinary excretion of biomarkers of choline metabolism also differed between third trimester pregnant and nonpregnant women. On average, third trimester pregnant women excreted 3.3 times more free choline ($P < 0.001$), 1.6 times more betaine ($P = 0.019$), 1.4 times more sarcosine ($P = 0.01$), 1.4 times more homocysteine ($P = 0.004$) and 1.7 times more methionine ($P < 0.001$) than nonpregnant women (Table 3).

Upon stratifying the cohort by choline intake (i.e., 480 or 930 mg choline/d), pregnancy affected the choline metabolites (data not shown) in a manner similar to that described in the preceding paragraphs.

TABLE 3 Estimated marginal means of biomarkers of choline metabolism among nonpregnant and third trimester pregnant women throughout the 12-wk study[1,2]

	Pregnancy status	
Variables	Nonpregnant women, *n* = 21	Pregnant women, *n* = 26
Plasma choline (μmol/L)	6.3 (5.6 – 6.9)	8.2 (7.6 – 8.7) [c]
Plasma betaine (μmol/L)	26 (23 – 31)	12 (10 – 14) [c]
Plasma DMG (μmol/L)	2.5 (2.1 – 3.0)	1.6 (1.3 – 1.9) [c]
Serum sarcosine (μmol/L)	1.1 (1.0 – 1.3)	0.6 (0.5 – 0.6) [c]
Serum Hcy (μmol/L)	6.0 (5.6 – 6.4)	4.0 (3.8 – 4.3) [c]
Serum methionine (μmol/L)	26 (25 – 27)	23 (22 – 24) [c]
Urinary choline (mg/d)	3.2 (2.3 – 4.4)	10.7 (8.1 – 14.1) [c]
Urinary betaine (mg/d)	8.1 (6.1 – 10.8)	12.9 (10.0 – 16.6) [a]
Urinary DMG (mg/d)	4.6 (3.4 – 6.1)	4.4 (3.4 – 5.8)
Urinary sarcosine (mg/d)	0.4 (0.3 – 0.5)	0.5 (0.4 – 0.6) [b]
Urinary Hcy (mg/d)	0.5 (0.4 -0.6)	0.7 (0.6 – 0.9) [b]
Urinary methionine (mg/d)	2.8 (2.3 – 3.4)	4.7 (4.2 – 5.3) [c]

[1]Data are presented as geometric mean (95% confidence intervals) throughout the 12-wk study

[2]Data were derived by using mixed linear models that tested the effect of pregnancy on the dependent variables as described in the text. Pregnant versus nonpregnant women: [a] indicates $P \leq 0.05$; [b] indicates $P \leq 0.01$; and [c] indicates $P \leq 0.001$. No significant interactions ($P > 0.05$) between pregnancy and choline intake were detected.

Abbreviations used: DMG, dimethylglycine; Hcy, total homocysteine.

Pregnancy x Time Interactions

Pregnancy interacted with time ($P < 0.05$) to influence the response of several circulating choline or 1-C metabolites (i.e., free choline, betaine, and homocysteine). Among third trimester pregnant women, circulating concentrations of free choline ($P < 0.001$; **Figure 2A**), betaine ($P < 0.001$; Figure 2C) and homocysteine ($P < 0.001$; **Figure 3C**) increased by 13-26% from the beginning (wk-0) to the end (wk-12) of the study. In contrast, circulating concentrations of free choline ($P = 0.9$; Figure 2B) and betaine ($P = 0.2$; Figure 2D) did not change among nonpregnant women and homocysteine decreased ($P < 0.001$; Figure 3D) by 16%. The effect of pregnancy on the trajectory of these metabolites during the second half of gestation is consistent with previous prospective studies [24, 25]. The increase in plasma free choline throughout the third trimester of pregnancy may be due to enhanced de novo choline synthesis, a reaction catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT). PEMT contains estrogen response elements and its expression is induced by estrogen [26] which rises throughout gestation. It also is worth noting that although plasma betaine and serum homocysteine rise throughout the third trimester, both variables remain lower ($P < 0.001$) than concentrations observed in nonpregnant control women.

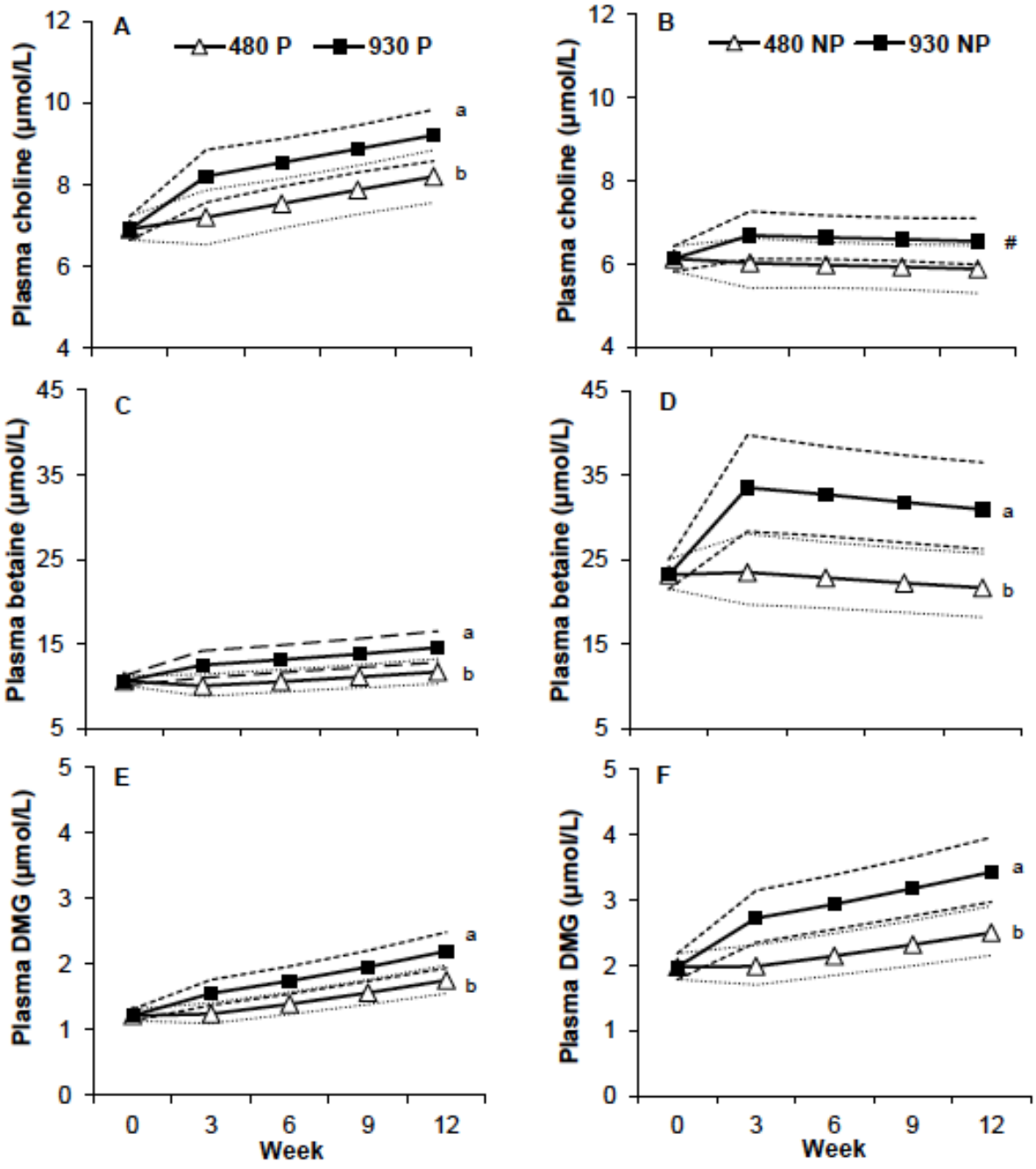
A
480 P
930 P
Plasma choline (µmol/L)
12
10
8
6
4
a
b
B
480 NP
930 NP
Plasma choline (µmol/L)
#
C
Plasma betaine (µmol/L)
45
35
25
15
5
a
b
D
Plasma betaine (µmol/L)
E
Plasma DMG (µmol/L)
5
4
3
2
1
0
F
Plasma DMG (µmol/L)
0
3
6
9
12
Week

Figure 2 legend

Estimated marginal means with 95% confidence intervals of plasma free choline, betaine and DMG among third trimester pregnant (A, C, E; $n = 26$) and nonpregnant women (B, D, F; $n = 21$) consuming 480 mg choline/d ($n = 13$ for pregnant group; $n = 11$ for nonpregnant group) or 930 mg choline/d ($n = 13$ for pregnant group; $n = 10$ for nonpregnant group) for 12-wk. All participants were included in the analysis, and the plotted data were estimated by using mixed linear regression models for third trimester pregnant and nonpregnant women separately. Wk-0 for pregnant women is wk-27 gestation. Different superscript letters indicate that the choline metabolite differed ($P < 0.05$) between the choline intake groups; superscript $^{\#}$ indicates that the choline metabolite tended to differ ($P = 0.08$) between the choline intake groups. Among pregnant women, plasma free choline ($P < 0.001$) and betaine ($P < 0.001$) increased throughout the 12-wk study; no effect of time ($P \geq 0.2$) was observed for these metabolites among nonpregnant women.

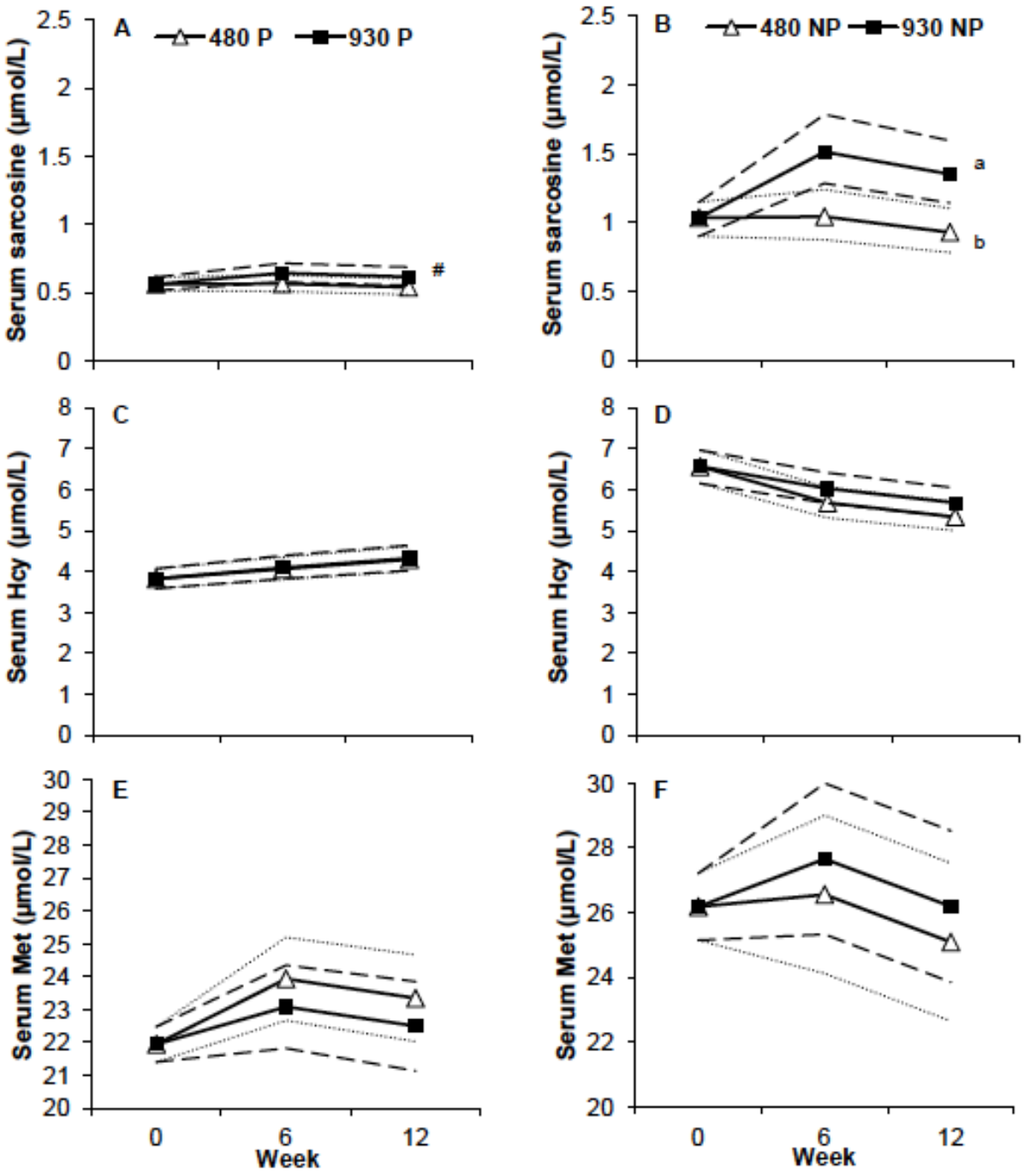
A
480 P
930 P
Serum sarcosine (μmol/L)
#
B
480 NP
930 NP
a
b
C
Serum Hcy (μmol/L)
D
E
Serum Met (μmol/L)
F
0
6
12
Week

Figure 3 legend

Estimated marginal means with 95% confidence interval of serum sarcosine, homocysteine (Hcy) and methionine (Met) among third trimester pregnant (A, C, E; n = 26) and nonpregnant women (B, D, F; n = 21) consuming 480 mg choline/d (n = 13 for pregnant group; n = 11 for nonpregnant group) or 930 mg choline/d (n = 13 for pregnant group; n = 10 for nonpregnant group) for 12-wk. All participants were included in the analysis, and the plotted data were estimated by using mixed linear regression models for third trimester pregnant and nonpregnant women separately as described in the text. Wk-0 for pregnant women is wk-27 gestation. Different superscript letters indicate that the choline metabolite differed ($P < 0.05$) between the choline intake groups; superscript $^{\#}$ indicates that the choline metabolite tended to differ ($P = 0.07$) between the choline intake groups. Serum homocysteine increased throughout the 12-wk study among third trimester pregnant women ($P < 0.001$) but decreased among nonpregnant women ($P < 0.001$).

Effects of choline intake on blood and urinary biomarkers of choline metabolism

Third trimester pregnant women

Consumption of 930 mg choline/d yielded 13% higher (P = 0.021) plasma choline (Figure 2A), 25% higher (P = 0.016) plasma betaine (Figure 2C), 25% higher (P = 0.012) plasma DMG (Figure 2E), and 14% higher (P = 0.07) serum sarcosine (Figure 3A) compared to consumption of 480 mg choline/d. Choline intake did not affect serum homocysteine (P = 0.9; Figure 3C) or serum methionine (P = 0.3; Figure 3E).

Choline intake did not affect urinary excretion of free choline (P = 0.3; **Figure 4A**), betaine (P = 0.9; Figure 4C), or DMG (P = 0.1; Figure 4E). However, consumption of 930 mg choline/d yielded 46% higher (P = 0.029) urinary sarcosine (**Figure 5A**) and 37% higher (P = 0.02) urinary methionine (Figure 5E) compared to consumption of 480 mg choline/d. Urinary homocysteine also tended to be higher (45%, P = 0.06) in the 930 versus 480 mg choline/d intake group (Figure 5C). These data collectively imply that a higher maternal choline intake enhances the metabolic flux of the methionine cycle, which generates both methionine and homocysteine.

Nonpregnant women

Consumption of 930 mg choline/d yielded 11% higher (P = 0.08) plasma choline (Figure 2B), 43% higher (P = 0.006) plasma betaine (Figure 2D), 37% higher (P = 0.004) plasma DMG (Figure 2F), and 46% higher (P = 0.004) serum sarcosine (Figure 3B) compared to consumption of 480 mg choline/d. Choline intake did not affect serum homocysteine (P = 0.2; Figure 3D) or serum methionine (P = 0.5; Figure 3F).

Choline intake did not affect urinary excretion of free choline (P = 0.8; Figure 4B), betaine (P = 0.6; Figure 4D), sarcosine (P = 0.3; Figure 5B), homocysteine (P = 0.7; Figure 5D), or methionine (P = 0.4; Figure 5F). However, consumption of 930 mg choline/d yielded 42% higher (P = 0.005) urinary DMG (Figure 4F) compared to consumption of 480 mg choline/d.

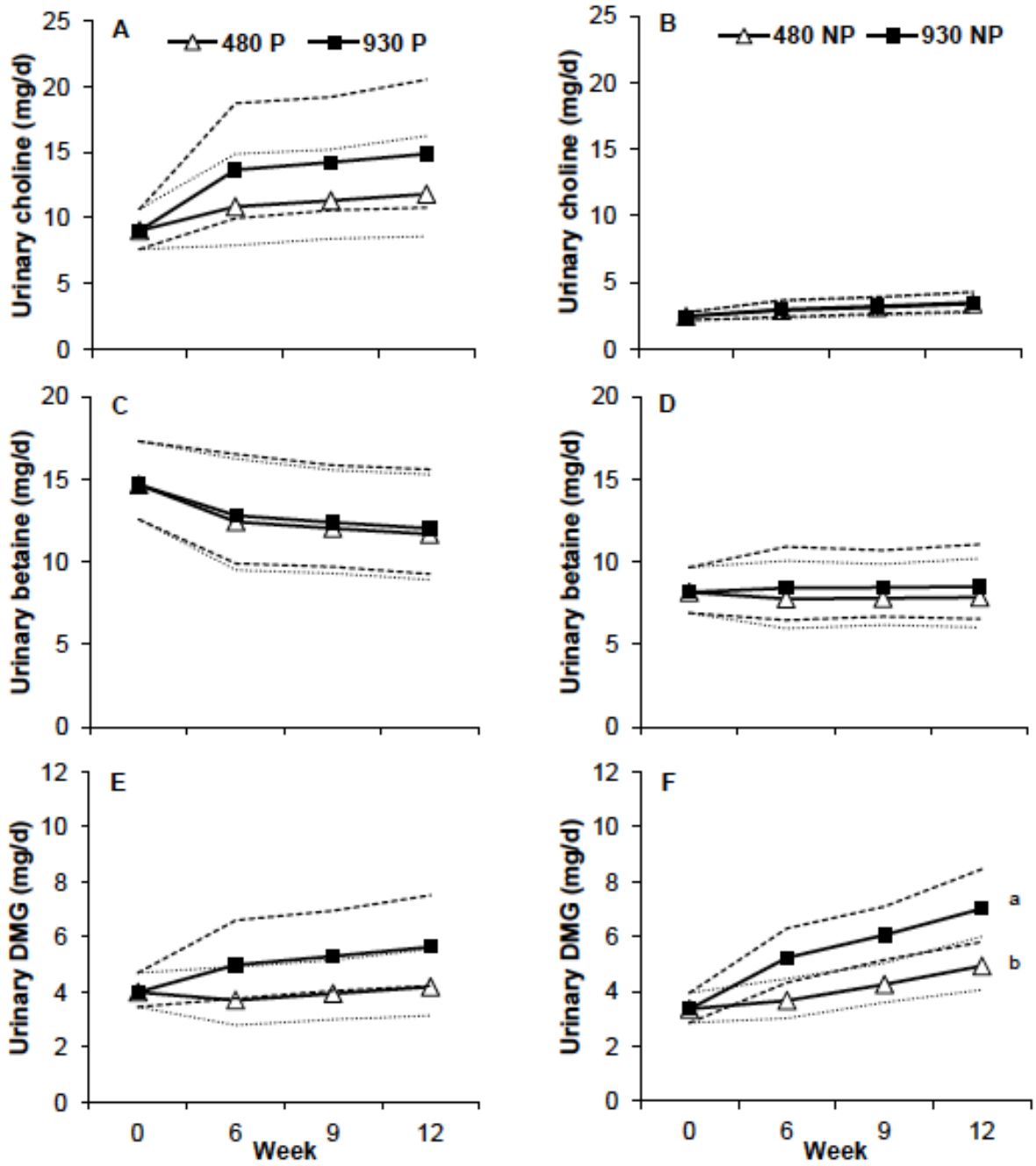

Figure 4 legend

Estimated marginal means with 95% confidence intervals of urinary free choline, betaine and DMG among third trimester pregnant (A, C, E; n = 26) and nonpregnant women (B, D, F; n = 21) consuming 480 mg choline/d (n = 13 for pregnant group; n = 11 for nonpregnant group) or 930 mg choline/d (n = 13 for pregnant group; n = 10 for nonpregnant group) for 12-wk. All participants were included in the analysis, and the plotted data were estimated by using mixed linear regression models for third trimester pregnant and nonpregnant women separately as described in the text. Wk-0 for pregnant women is wk-27 gestation. Different superscript letters indicate that the choline metabolite differed ($P < 0.05$) between the choline intake groups.

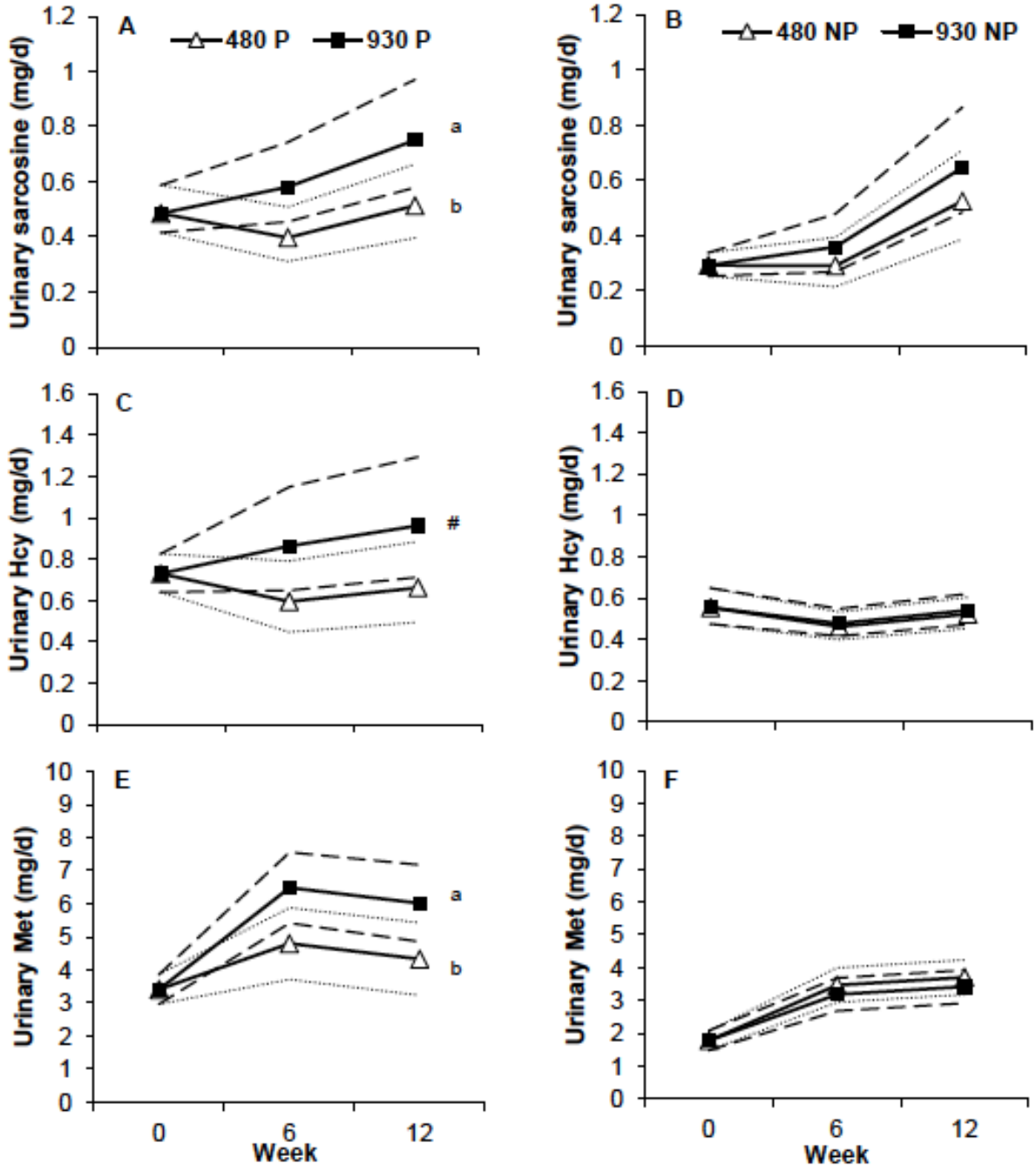
A
480 P
930 P
Urinary sarcosine (mg/d)
a
b
B
480 NP
930 NP
Urinary sarcosine (mg/d)
C
Urinary Hcy (mg/d)
#
D
Urinary Hcy (mg/d)
E
Urinary Met (mg/d)
a
b
F
Urinary Met (mg/d)
0
6
12
Week

Figure 5 legend

Estimated marginal means with 95% confidence intervals of urinary sarcosine, homocysteine (Hcy) and methionine (Met) among third trimester pregnant (A, C, E; *n* = 26) and nonpregnant women (B, D, F; *n* = 21) consuming 480 mg choline/d (*n* = 13 for pregnant group; *n* = 11 for nonpregnant group) or 930 mg choline/d (*n* = 13 for pregnant group; *n* = 10 for nonpregnant group) for 12-wk. All participants were included in the analysis, and the plotted data were estimated by using mixed linear regression models for third trimester pregnant and nonpregnant women separately as described in the text. Wk-0 for pregnant women is wk-27 gestation. Different superscript letters indicate that the choline metabolite differed ($P < 0.05$) between the choline intake groups; superscript $^{\#}$ indicates that the choline metabolite tended to differ ($P = 0.06$) between the choline intake groups.

Effects of pregnancy and choline intake on plasma and urinary TMAO

At wk-12, plasma TMAO concentration (P = 0.024; **Figure 6A**) and urinary TMAO excretion (P = 0.008; Figure 6B) were higher in the 930 versus 480 mg choline/d intake group. However, the difference in plasma TMAO between choline intake groups was mainly driven by 3 out of 24 participants. Exclusion of these participants from the statistical analysis attenuated the effects of choline intake on plasma TMAO response (2.9 ± 0.5 vs. 2.2 ± 0.4 μmol/L; P = 0.07). Pregnancy did not alter plasma TMAO or urinary TMAO excretion nor did it interact with choline intake to affect TMAO abundance (data not shown; P > 0.1). Notably, a broad range of plasma TMAO concentration and urinary TMAO excretion was observed at wk-12 with choline intakes of either 480 mg/d (0.7-7.7 μmol/L for plasma TMAO; 1.3-129 mg/day for urinary TMAO) or 930 mg/d (0.6-20.3 μmol/L for plasma TMAO; 0.2-162 mg/d for urinary TMAO excretion). The large inter-individual variation in plasma and urinary TMAO under conditions of controlled dietary intake (choline and alternative trimethylamine-containing species) is consistent with individual differences in the composition of colonic microflora. As such, the production of TMAO in response to increased choline intake is largely a function of the gut microbiome.

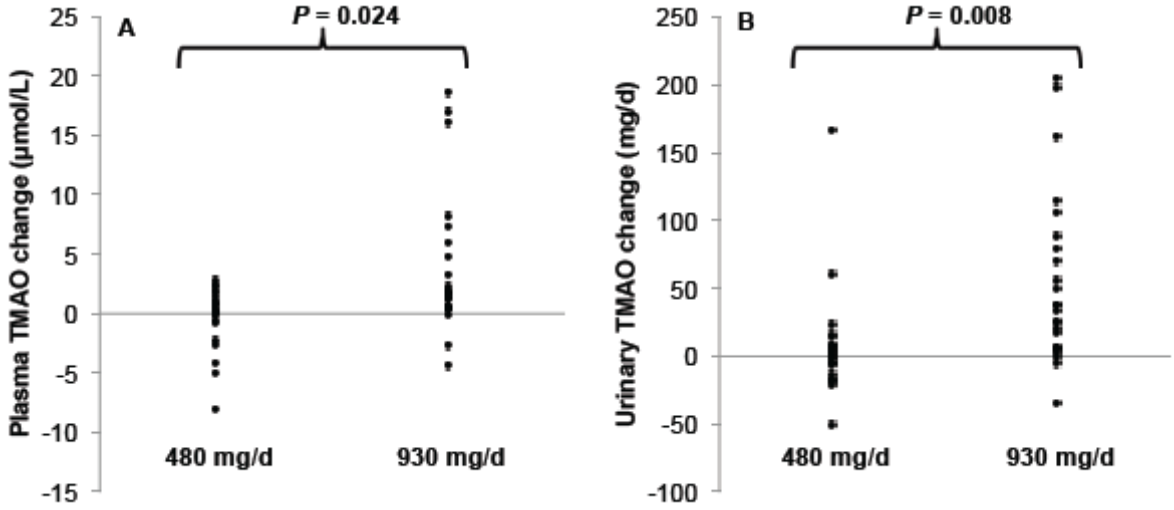

Figure 6 legend

Scatter plot of the change (wk-12 *minus* wk-0) in plasma TMAO concentration (A) and urinary TMAO excretion (B) among third trimester pregnant (n = 26) and nonpregnant (n = 21) women consuming 480 (n = 13 for pregnant group; n = 11 for nonpregnant group) or 930 (n = 13 for pregnant group; n = 10 for nonpregnant group) mg choline/d for 12-wk. Data were analyzed using 2-way ANOVA with the change through time as the dependent variable, choline intake, pregnancy as well as the baseline measure (wk-0) as the independent variables.

Clinical and biochemical measures of placenta and infants at birth

Three pregnant women delivered within the final week of the study, and the others delivered 0 to 24 days after wk-12. The median (range) interval between the end of the study and delivery was 10 (-5, 24) days for 480 mg choline/d group, which did not significantly differ (Kolmogorov–Smirnov test, $P = 0.16$) from 4 (-6, 24) days for 930 mg choline/d group.

The mean gestational week at birth was 40 and did not differ between the choline intake groups. Twenty-seven percent of the newborns were female and the gender distribution between groups was balanced. Key fetal and placental parameters (**Table 4**) were within normal range and did not differ between choline intake groups ($P > 0.05$).

Placental choline and betaine concentrations did not differ ($P > 0.05$) between choline intake groups (Table 4). However, the higher maternal choline intake tended to increase placental acetylcholine accumulation ($P = 0.089$). To further elucidate the effects of maternal choline intake on acetylcholine metabolism, we examined the mRNA abundance of *ChAT*, which catalyzes the synthesis of acetylcholine from choline. We found that transcript abundance was elevated ($P = 0.022$) in the 930 mg choline/d intake group suggesting that the higher maternal choline intake enhances the placental synthesis of acetylcholine which may facilitate amino acid transport, cellular differentiation and parturition [27, 28]. DMG was not detected in the placental tissue.

Consistent with previous reports [29, 30], cord plasma choline was 4.4 times higher (35 ± 10 vs. 8 ± 2 μmol/L; $P < 0.001$) and cord plasma betaine was 3 times higher (30 ± 7 vs. 10 ± 5 μmol/L; $P < 0.001$) than maternal plasma concentrations at delivery. However, in contrast to prior work showing higher circulating DMG in cord versus maternal plasma [29-31], we found 29% lower circulating DMG in cord versus maternal plasma (2.0 ± 1.0 vs. 2.8 ± 1.0 μmol/L; $P < 0.001$). Notably, maternal plasma DMG in the present study was

approximately 56% higher than previous findings [28, 29] possibly due to supplementation of the maternal diet with extra choline (i.e, 100 or 550 mg choline/d).

Although maternal choline intake did not affect concentrations of choline or betaine in cord venous plasma ($P > 0.1$), the higher maternal choline intake (930 versus 480 mg choline/d) led to a doubling of cord plasma DMG ($P = 0.002$; Table 4), the metabolite produced when choline is used as a methyl donor.

TABLE 4

Physiological and biochemical parameters of placenta and neonates of pregnant women consuming 480 or 930 mg choline/d throughout the third trimester[1,2]

	Choline Intake	
Variables	480 mg/d, *n* = 13	930 mg/d, *n* = 13
Cesarean section (N)	3	2
Gestational age at birth (week)	40 (38-41)	40 (39-40)
Total weight gain throughout the study (kg)	6.4 ± 2.7	6.0 ± 2.2
Infant gender (female : male)	3 : 10	4 : 9
Infant birth weight (kg)	3.4 ± 0.4	3.5 ± 0.3
Infant Length (cm)	50.4 ± 2.0	50.9 ± 2.3
Infant head circumference (cm)	34.4 ± 1.1	34.3 ± 1.3
Infant Apgar scores	8.7 ± 0.5; 9 ± 0	8.2 ± 1.4; 8.8 ± 0.6
Placenta weight (gram)	538 ± 108	529 ± 102
Placenta length (cm)	15.8 ± 2.3	16.4 ± 1.2
Placenta width (cm)	19.7 ± 2.7	18.9 ± 1.9
Placenta depth (cm)	1.6 ± 0.3	1.7 ± 0.4
Placenta choline (nmol/g tissue)	915 ± 231	941 ± 309
Placenta betaine (nmol/g tissue)	37 ± 6	37 ± 10
Placenta acetylcholine (nmol/g tissue)	65 ± 25	88 ± 38 $^{+}$
Placental *ChAT* mRNA expression[3]	0.6 ± 0.2	1.4 ± 0.3 ^{a}
Cord plasma choline (μmol/L)	37.3 ± 13	32.5 ± 7.5
Cord plasma betaine (μmol/L)	29.9 ± 6.1	29.4 ± 7.5
Cord plasma DMG (μmol/L)	1.3 ± 0.5	2.6 ± 0.9 ^{b}

[1]Data are presented as mean ± SD or (range).

[2]Data were analyzed with 1-way ANOVA; *n* = 11-13 per group; ^{a} indicates $P \leq 0.05$; ^{b} indicates $P \leq 0.01$; $^{+}$ indicates $0.05 < P < 0.1$

[3]Data are presented as the fold-change of the housekeeping gene: glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*

Abbreviations used: *ChAT*, choline acetyltransferase; DMG, dimethylglycine

DISCUSSION

This highly controlled feeding study sought to quantify the effects of pregnancy and maternal choline intake on maternal and fetal biomarkers of choline metabolism. Two main findings emerged: (1) pregnancy markedly alters biomarkers of choline metabolism, and (2) a higher maternal choline intake increases the use of choline as a methyl donor in both maternal and fetal compartments.

Circulating choline-derived methyl donors are markedly lower in third trimester pregnant women as compared to nonpregnant women

In the present study with controlled choline intake, plasma betaine and DMG were 40-55% lower in third trimester pregnant than nonpregnant women; a finding that is consistent with previous observational reports [24, 29, 30]. We also report for the first time that serum sarcosine is 49% lower in third trimester pregnant than nonpregnant women. Although hemodilution can contribute to reductions in circulating metabolites during pregnancy, expansion of maternal plasma cannot fully explain the lower concentrations of these methyl donors. Maternal plasma volume begins to increase at wk-10 gestation and expands an average of 45% to provide for the greater circulatory needs of the maternal/fetal organs [32]. As such, hemodilution would be expected to yield a 30% reduction in the circulating metabolites, not the observed 40-55% reduction.

An additional explanation is the increased use of choline-derived methyl groups by pregnant women. Accelerations in 1-C metabolism transpire during pregnancy to support cellular proliferation (e.g., nucleotide biosynthesis) and the establishment of epigenetic methylation marks on newly formed cells [33]. Following the use of betaine as a methyl donor in the formation of the SAM-precursor methionine, the methyl groups associated with DMG and subsequently methylglycine (sarcosine) are used in folate-mediated 1-C metabolism for the synthesis of purines, thymidylate, or methionine.

Notably, mean serum folate concentrations were elevated (i.e., > 20 ng/mL) among the pregnant women at baseline (wk-0) and throughout the study due to their consumption of a folic acid containing prenatal vitamin. Thus, although folate and choline are highly interrelated, the diminished circulating concentrations of choline-derived methyl donors among folate replete pregnant women suggests that extra folate (i.e., folic acid) cannot compensate for the higher use of choline as a methyl donor. This may be due to the fact that folate is predominately a carrier of methyl groups whereas choline is a source.

Obligatory losses of urinary choline and betaine were ~2-4 times higher among pregnant women

Pregnant women excreted ~ 4 times more free choline than nonpregnant women at baseline (wk-0) and throughout the study. A greater excretion of free choline has also been demonstrated in hyperlipoproteinemic adults [34], a condition that transpires during pregnancy [35]. As lipoproteins contain phosphatidylcholine, the greater urinary excretion of free choline by pregnant women (relative to nonpregnant women) may be a consequence of enhanced uptake and catabolism of lipoprotein-derived phosphatidylcholine in kidney. The relatively strong relationship between the urinary excretion of free choline and serum total cholesterol at wk-0 (Pearson's coefficient $r = 0.6$; $P = 0.004$) and wk-12 ($r = 0.4$; $P = 0.016$) is consistent with this metabolic scenario.

To the best of our knowledge, this is the first report of greater urinary betaine excretion in pregnant versus nonpregnant women. Normally, only small amounts of betaine are excreted in the urine. However, during pregnancy, the marked increase in plasma volume and glomerular filtration likely contributed to the 2 times greater urinary loss of betaine in third trimester pregnant versus nonpregnant women.

The markedly diminished circulating concentrations of choline-derived methyl donors among pregnant women coupled with their greater urinary excretion of choline and betaine

(~13 mg/d when expressed as a choline equivalent) imply that an increment of 25 mg choline/d (450 versus 425 mg choline/d) is insufficient to meet the choline demands of pregnancy.

A higher maternal choline intake results in enhanced use of choline as a methyl donor

A higher choline intake resulted in the greater maternal use of choline as a methyl donor as evidenced by the higher concentrations of DMG and sarcosine (intermediates formed in the demethylation of choline). The higher concentrations of DMG and sarcosine on the higher choline intake level are also consistent with an increased availability of carbons for folate-mediated 1-C metabolism. Notably, the higher intake level did not alter the urinary excretion of free choline and betaine, which implies that a doubling of the choline AI does not exceed the cells capacity to use the extra choline as a methyl donor.

Although maternal choline intake did not influence neonatal circulating concentrations of free choline or betaine, DMG was two times higher in the neonates of mothers consuming 930 versus 480 mg choline/d. As DMG was not detected in the placental tissue samples, the DMG in fetal circulation was most likely of fetal origin and generated upon the use of choline and/or betaine as methyl donors. To the best of our knowledge, this is the first human study to demonstrate an effect of maternal choline intake on neonatal biomarkers of choline metabolism.

Choline-derived methyl groups are needed for DNA methylation, which is essential for developmental processes including genomic imprinting and maintenance of genome stability [36]. The increased fetal and maternal use of choline as a methyl donor in the 930 versus 480 mg choline/d intake group is likely to have functional consequences that are mediated through epigenetic mechanisms. The genomic consequences of the higher choline intake are currently being explored via measurements of placental and blood leukocyte DNA methylation (global and site specific) as well as gene expression analyses.

Conclusions and Implications of Study Findings

In sum, third trimester pregnant women have substantially lower circulating concentrations of choline-derived methyl donors even under folate-replete conditions. Coupled with their increased obligatory loss of urinary choline and betaine (~ 13 mg/d as choline equivalents), these data suggest that an additional 25 mg choline/d is insufficient in meeting the choline demands of third trimester pregnant women. Moreover, the study findings demonstrate that maternal choline intake modifies both maternal and fetal biomarkers of choline metabolism with evidence of greater use of choline as a methyl donor on a higher choline intake.

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CHAPTER 3

Pregnancy alters choline metabolism in women consuming methyl-d9-choline

ABSTRACT

Background: Little is known about choline metabolism in human pregnancy.

Objective: This study sought to test the hypothesis that pregnancy alters the metabolic use of orally consumed *methyl*-d9-choline.

Design: Healthy third trimester pregnant (n = 26, wk 27 gestation) and nonpregnant (n = 21) women consumed ~22% of their total choline intake (480 or 930 mg/d) as *methyl*-d9-choline for the last 6-wk of a 12-wk feeding study.

Results: At study-end, a lower ($P < 0.001$) plasma enrichment ratio of d9-betaine:d9-phosphatidylcholine (PC) was observed in pregnant vs. nonpregnant women, implying preferential partitioning of choline into the CDP-choline pathway relative to betaine synthesis. Nonetheless, the use of betaine for methionine synthesis was enhanced in pregnant (vs. nonpregnant) women, as suggested by a higher ($P < 0.001$) plasma enrichment ratio of d3-methionine:(d3+d9)-betaine. Pregnant (vs. nonpregnant) women also used more choline derived methyl groups for PC synthesis via phosphatidylethanolamine *N*-methyltransferase (PEMT) to support their larger pool size as indicated by a comparable increase in PEMT-PC enrichment across the last 3-wk of the study. The hydrolysis of PEMT-PC to free choline was also enhanced during pregnancy as suggested by a higher plasma enrichment ratio of d3-choline:d3-PC ($P < 0.001$). Notably, d3-PC enrichment increased ($P < 0.05$) incrementally from the maternal to placental to fetal compartment implying a selective transfer of PEMT-PC to the fetus.

Conclusions: These data demonstrate, for the first time in humans, that both pathways for PC synthesis are elevated in late pregnancy with evidence of preferential transfer of PEMT-PC towards the fetus.

INTRODUCTION

Pregnancy is a physiologic state characterized by rapid cellular proliferation and accelerated one-carbon metabolism [1, 2], both of which increase the demand for the essential nutrient choline. Through the cytidine diphosphate-choline (CDP-choline) pathway, choline is used to produce phosphatidylcholine (PC), a constituent of all cellular membranes and a requisite for tissue expansion. Through its oxidation to betaine, choline serves as a methyl donor in one-carbon metabolism and is used to synthesize methionine and *S*-adenosylmethionine (SAM), the principal methyl donor in mammalian cells. Notably, a main consumer of SAM is phosphatidylethanolamine *N*-methyltransferase (PEMT), which catalyzes the denovo biosynthesis of PC (and choline) from phosphatidylethanolamine (PE).

Dietary recommendations for choline, in the form of an adequate intake (AI), were established by the Institute of Medicine (IOM) for the first time in 1998 [3]. The AI of 450 mg choline/d for pregnant women is only slightly higher than the AI of 425 mg choline/d for nonpregnant women, and may not be sufficient in meeting the demand for choline during late pregnancy [4]. Specifically, in a 12-wk controlled feeding study conducted by our research group, third trimester pregnant women consuming a level of choline intake approximating the AI (i.e. 480 mg choline/d) exhibited depletion of choline derived methyl donors as compared to nonpregnant women with an equivalent choline intake [4]. In addition, consumption of 930 mg choline/d partially normalized circulating concentrations of choline derived methyl donors [4], increased the use of choline as a methyl donor in maternal and fetal derived tissues [4, 5], and yielded lower circulating concentrations of the stress hormone cortisol in fetal cord blood [5].

In our study by Yan et al [4], pregnant and nonpregnant women consumed ~22% of their total choline intake (either 480 or 930 mg choline/d) as *methyl*-d9-choline during the last 6 wk of the 12 wk study. As such, the present study seeks to extend this work by employing stable isotope methodology to quantify the effect of pregnancy and choline intake on the metabolic fate of *methyl*-d9-choline.

STUDY PARTICIPANTS AND METHODS

Study participants

Healthy third trimester singleton pregnant women (gestational wk 26-29) aged ≥ 21 y and nonpregnant women (n=21) were recruited from the Ithaca, NY area from January 2009–October 2010 as described by Yan et al [4]. During the screening phase, all study volunteers completed a health history and questionnaire and provided a blood sample for blood chemistry profiling and a complete blood count. Entry into the study was contingent upon (i) good health status (i.e., no chronic diseases, normal kidney and liver function, and non-anemic), (ii) no drug or alcohol use, and (iii) willingness to comply with study protocol [4]. Additional inclusion and exclusion criteria were detailed previously [4].

The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and at the Cayuga Medical Center (the hospital where pregnant participants delivered their babies; Ithaca, NY). Written informed consent was obtained from all participants prior to their entry into the study.

Study Design, Diet, and Supplements

Design

This was a 12-wk controlled feeding study in which pregnant women (n = 26; wk 26-29 gestation) and nonpregnant women were randomized to consume either 480 (~ choline AI) or 930 mg choline/d. The total choline intake was obtained from the diet (380 mg/d) plus supplemental choline chloride (100 mg choline/d for the 480 mg/d intake level; 550 mg choline/d for the 930 mg/d intake level). During the last 6 wk of the 12 wk study, ~22% of their total choline intake was consumed as supplemental *methyl*-d9-choline (100 or 200 mg choline/d). For the period between completion of the 12-wk study and delivery of the infant, pregnant women who had not yet delivered continued to consume their assigned choline supplement. Maternal blood (wk 9, 10 and 12) and urine (wk 9 and 12) were collected

throughout the feeding phase of the study; a maternal blood sample, placenta tissue and cord blood were obtained at delivery [4, 5].

Diet

Throughout the 12 wk study, participants consumed a 7-day cycle menu as detailed previously [4]. The study diet provided a total of 380 mg choline/d: 236 mg were derived from lipid soluble forms and 142 mg were derived from water-soluble forms [4]. The diet also provided 100 mg betaine/d. Food was prepared in the Francis A. Johnston and Charlotte M. Young Human Metabolic Research Unit (HMRU) at Cornell University. Study participants consumed at least one meal/d at the on-site location (i.e., HMRU) during the week under the supervision of study personnel; all other food and beverages were provided as take-aways, and study participants were required to verify consumption of each food item by completing a daily checklist provided by the investigators.

Supplements

To achieve the target choline intakes of 480 or 930 mg/d, unlabeled choline supplementation prepared with commercially available choline chloride (Balchem, New Hampton, NY) was consumed for the first 6 wk. During the last 6-wk of the study (wk 6-12), participants obtained ~22% of the their target choline intake (100 or 200 mg choline/d) through deuterium labeled choline supplementation prepared from commercial available *methyl*-d9-choline (Cambridge Isotope Laboratories Inc, Andover, MA). Specifically, in the 480 mg choline/d intake group, choline was derived from the diet (380 mg/d), and a single bolus of *methyl*-d9-labeled supplemental choline chloride (100 mg/d). In the 930 mg choline/d intake group, choline was derived from the diet (380 mg/d), and a single bolus of unlabeled choline chloride (350 mg/d) mixed with the *methyl*-d9-labeled choline chloride (200 mg/d). Choline supplements were prepared by study personnel as detailed previously [4].

In addition to the supplemental choline, a prenatal multivitamin (Pregnancy Plus, Fairhaven Health, LLC) and docosahexaenoic acid (DHA; 200 mg, Neuromins, Nature's Way Products) were provided daily, and a potassium and magnesium supplement (General Nutrition Corp) was provided three times per week. Supplements were consumed under the supervision of study personnel at the on-site meal; otherwise, supplements were provided in plastic bags along with take-away meals and participants were instructed to consume the supplements with a meal of their choice.

Compliance

Overall, the study protocol was well tolerated with 92% of the participants completing the study regimen (21 of 22 nonpregnant women, 26 of 29 pregnant women). Among 26 pregnant women completing the regimen, twenty-two completed the 12 wk study and four women completed 10 wk study (due to early delivery). Additional reasons for stopping the study included nausea, food dislikes, and personal challenges. The detailed flow of study participants through the screening and intervention phases has been reported previously [4].

Sample collection and processing

Fasting maternal blood samples throughout the 12-wk study were collected into serum separator gel and clot-activator tube (SST Vacutainer; Becton, Dickinson and Company) and EDTA coated tubes (Vacutainer). Serum and plasma were obtained and stored at -80 °C as previously described [4, 6, 7]. 24-h urine samples were collected throughout the 12-wk study and were processed and stored at -80 °C as described previously [4].

Maternal delivery blood samples were collected into EDTA coated tubes at the hospital within 24 h before delivery. Cord venous blood samples were also collected into EDTA-coated tubes at the time of delivery. Maternal delivery blood and cord venous blood were processed for plasma and stored at -80 °C. In addition, the placenta was obtained at

delivery, processed at the hospital within 90 min of delivery, and stored at -80 °C as previously described [4, 5].

Measurements of plasma, urinary, and placental choline metabolites

Plasma and placental tissue PC (d0, d3, d6, and d9) and SM (d0, d3, d6, and d9) were extracted and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described by Koc et al [8] with modifications based on our instrumentation [9]. Ions with m/z ratio of 184 were monitored for unlabeled metabolites (d0-PC and d0-SM) and ions with m/z ratios of 187, 190, and 193 were monitored for d3 labeled metabolites (d3-PC and d3-SM), d6 labeled metabolites (d6-PC and d6-SM), and d9 labeled metabolites (d9-PC and d9-SM), respectively.

Plasma, placental tissue, and urinary free choline (d0, d3, d6, and d9), betaine (d0, d3, d6, and d9), and dimethylglycine (d0, d3, and d6) were extracted and quantified by LC-MS/MS according to the method of Holm et al [10] with modifications based on our instrumentations [4, 9]. The ions with m/z ratios monitored for each metabolite was as following: d0-choline, 104 → 60; d3-choline, 107 → 63; d6-choline, 110 → 66; d9-choline, 113 → 69; d0-betaine, 118 → 59; d3-betaine 121 → 62; d6-betaine, 124 → 65; d9-betiane, 127 → 68; d0-DMG, 104 → 58; d3-DMG, 107 → 61; d6-DMG, 110 → 64.

Serum and urinary sarcosine (d0 and d3) and methionine (d0 and d3) were extracted and quantified by GC-MS as described previously [11, 12].

Urinary *S*-adenosylmethionine (d0 and d3) was extracted and quantified by LC-MS/MS as described by Kim et al [13] with modifications based on our instrumentation [5].

Rationale for using *methyl*-d9-choline as a tracer

As shown **in Figure 1**, the choline tracer used in the present study, *methyl*-d9-choline is labeled with deuterium on all three methyl groups. Therefore, it traces the fate of the intact molecule (d9-choline metabolites) and of its methyl groups (predominately d3-choline metabolites) [9]. The administered *methyl*-d9-choline can enter the CDP-choline pathway to produce d9-PC or it can be oxidized to d9-betaine, which donates a methyl group to homocysteine, producing d6-DMG and d3-methionine. D3-methionine serves a precursor to d3-SAM, which can be used by PEMT to sequentially methylate phosphatidylethanolamine (PE) forming d3-PC, d6-PC, and small amounts of d9-PC [4]. Under the current labeling strategy, the amount of PEMT derived d9-PC (~0.02% of plasma total PC) is lower than the detection limits (~ 0.1% of plasma total PC). Therefore, we consider that all the detected d9-PC is derived from CDP-choline pathway. Hydrolysis of d3-PC will generate d3-choline as well as d3-betaine, d3-methinone, d3-DMG, and d3-sarcosine when d3-choline is used as a methyl donor.

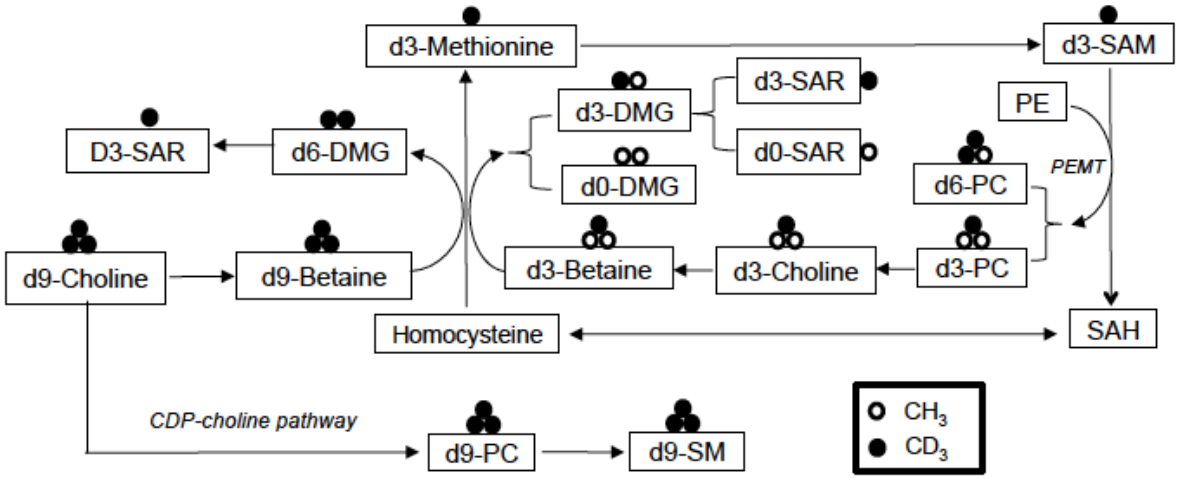

Figure 1 legend

Deuterium labeled *methyl*-d9-choline traces the distribution of orally consumed choline and its methyl groups. Black circles indicate deuterium labeled methyl groups; white circles indicated unlabeled methyl groups. Abbreviations: DMG, dimethylglycine; SAR, sarcosine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SM, sphingomyelin; PEMT, phosphatidylethanolamine *N*-methyltransferase.

Isotopic enrichment [labeled metabolite/(labeled + unlabeled metabolite)] was examined in blood, urine, and placental tissue to monitor the flow of dietary choline (d9-choline metabolites) and its methyl groups (d3-choline metabolites) through choline metabolic pathways. The isotopic enrichment percentage was calculated using the peak area under the chromatography curve of the labeled metabolite divide by the total area of all isotopomers of the metabolite. For example, the enrichment percentage of d3-PC = peak area of d3-PC / peak area of (d0+d3+d6+d9)-PC × 100%. The enrichment of d3-PC indicates the proportion of PEMT derived d3-PC in plasma total PC pool. Metabolite enrichment ratios were examined as indicators of orally consumed choline use through a specific portion of a pathway (enrichment of product/ enrichment of precursor) or its partitioning between pathways.

In addition, based on the principle of Mass Isotopomer Distribution Analysis (MIDA) [14, 15], we can use the mass spectra abundances of d0, d3, d6, and d9 choline isotopomers to delineate the effect of pregnancy and choline intake on the activity of PEMT mediated PC synthesis (which mainly occurs in the liver). For this purpose, the equation defined by Foster et al [16] for calculating the fractional synthesis rate (i.e., the fraction of total PC which is replaced by PEMT-PC using choline derived methyl groups) was modified to accommodate the longitudinal design of the present study as depicted in Equation 1:

The incremental labeling of plasma PC pool with PEMT-PC between time points 1 and 2

$$= \frac{E_{PEMT\_t2} - E_{PEMT\_t1}}{(E_{SAM\_t1} + E_{SAM\_t2})/2} \qquad (1)$$

E_{PEMT_t1} and E_{PEMT_t2} were the plasma enrichment of PEMT-PC (d3+d6-PC) at time point 1 and 2; E_{SAM_t1} and E_{SAM_t2} were the hepatic enrichment of d3-SAM at time point 1 and 2. Equation 1 was further modified to adjust for differences between the weeks in PC pool size as depicted in Equation 2:

The incremental labeling of plasma PC pool with PEMT-PC between time points 1 and 2

$$= \frac{E_{PEMT\_t2} \times {Pool_{PC\_t2}}/{Pool_{PC\_t1}} - E_{PEMT\_t1}}{(E_{SAM\_t1}+E_{SAM\_t2})/2} \quad (2)$$

$Pool_{PC\_t1}$ and $Pool_{PC\_t2}$ were the concentrations of plasma PC at time point 1 and 2. The calculated values from equation 2 represent the incremental labeling of the plasma PC pool with PEMT-PC and indicate the flux of choline derived methyl groups through the PEMT pathway.

This strategy requires an estimation of the hepatic enrichment of the precursor molecule, d3-SAM. The hepatic enrichment of d3-SAM can be calculated using the plasma enrichment of its products, d3-PC and d6-PC, which were quantified in the present study. The equation for the calculation of d3-SAM is as follows [15]:

$$\text{Hepatic d3-SAM enrichment} = \frac{E_{d6-PC}}{E_{d6-PC} + E_{d3-PC}} \quad (3)$$

$E_{d3\text{-}PC}$ and $E_{d6\text{-}PC}$ were the quantified enrichment of d3-PC and d6-PC. In our study, the calculated hepatic d3-SAM enrichment was highly correlated (Pearson's correlation coefficient $r = 0.74$, $P < 0.001$) with the measured urinary d3-SAM enrichment.

Statistical analysis

Twenty-one nonpregnant and twenty-two third trimester pregnant women completed the 12-wk study and were included in the statistical analysis.

Plots and histograms of the residuals were used to assess normality and variance homogeneity of dependent variables. All dependent variables were fit the assumption of the analysis of variance model.

To delineate the effect of pregnancy on study-end (wk 12) concentrations of the choline metabolites and their enrichment, a 2-factor ANOVA (choline intake and pregnancy)

including the interaction term (pregnancy × choline intake) was conducted. If the interaction term was significant at $P \leq 0.05$, data were stratified by choline intake, and the effect of pregnancy on metabolite concentrations was examined for women consuming 480 or 930 mg choline/d, separately. Because pregnancy is a physiologic state, baseline values were not entered as covariates into the statistical model.

To evaluate the main effect of choline intake on the dependent variables at wk 12, we conducted a 1-factor ANOVA (choline intake) for pregnant and nonpregnant women, separately. In addition, for the choline metabolite concentrations, baseline (wk 0) values were included in the model as a covariate.

To test for differences in the maternal delivery, placental, and fetal dependent variables between choline intake groups, a 1-factor ANOVA (choline intake) was performed. The time interval between completion of the 12-wk study and delivery of the infant was included as a covariate in the model if it was a significant predictor of the dependent variable. Paired t tests were performed to compare corresponding variables in maternal delivery blood, placental tissue, and fetal cord blood.

Data are presented as means ± SEM (unless noted otherwise) and were analyzed by SPSS software (version 20; SPSS Inc, Chicago, IL). Differences were considered to be significant at $P \leq 0.05$, whereas a $P < 0.10$ was indicative of trends.

RESULTS

Characteristics of study population at the study-end (wk 12)

Table 1 shows the plasma and urinary choline metabolites and biochemical indices related to one-carbon metabolism at study-end (wk 12). Compared to nonpregnant women, third trimester pregnant women had 47 % higher ($P < 0.001$) plasma choline, 61% higher ($P < 0.001$) plasma PC, but lower (11-61%; $P < 0.01$) circulating concentrations of betaine, DMG, sarcosine and methionine. In addition, the obligatory losses of urinary choline and betaine were 2 ~ 5 times greater ($P \leq 0.028$) among pregnant versus nonpregnant women.

Among nonpregnant women, consumption of 930 (versus 480) mg choline/d yielded higher (29 - 67%, $P < 0.02$) circulating concentrations of betaine, DMG and sarcosine as well as greater excretion of urinary DMG (70%, $P = 0.009$). Among pregnant women, consumption of 930 (versus 480) mg choline/d tended to yield 48% higher plasma betaine ($P = 0.059$), 47% higher plasma DMG ($P = 0.057$) and 140% greater urinary sarcosine ($P = 0.078$).

TABLE 1: Blood and urinary concentrations of choline metabolites and biochemical indices related to one-carbon metabolism at study-end (wk 12) in nonpregnant and third trimester pregnant women consuming 480 or 930 mg choline/d for 12 wks[1]

	Nonpregnant women, *n* = 21			Third trimester pregnant women, *n* = 22		
Variables	480 mg/d	930 mg/d [3]	All [2]	480 mg/d	930 mg/d [3]	All [2]
Plasma choline (μmol/L)	5.7 ± 0.4	6.2 ± 0.3	6.0 ± 0.2	8.0 ± 0.5	9.6 ± 0.7	8.8 ± 0.5 ***
Plasma betaine (μmol/L)	21 ± 2.8	35 ± 3.3 [a]	28 ± 2.4	12 ± 0.5	18 ± 3.0 #	14 ± 1.4 ***
Plasma DMG (μmol/L)	2.8 ± 0.8	3.6 ± 0.5 [b]	3.2 ± 0.4	1.7 ± 0.2	2.5 ± 0.4 #	2.0 ± 0.2 **
Serum sarcosine (μmol/L)	0.9 ± 0.1	1.4 ± 0.1 [c]	1.2 ± 0.08	0.56 ± 0.05	0.65 ± 0.04	0.6 ± 0.03 ***
Serum methionine (μmol/L)	25 ± 1.2	26 ± 1.2	26 ± 0.8	24 ± 0.9	22 ± 0.7	23 ± 0.6 **
Serum homocysteine (μmol/L)	5.6 ± 0.3	5.8 ± 0.3	5.6 ± 0.2	4.6 ± 0.3	4 ± 0.2	4.4 ± 0.2 ***
Serum folate (ng/mL)	30 ± 2.4	26 ± 1.6	28 ± 1.4	29 ± 2.8	26 ± 2.3	27 ± 1.8
Plasma PC (μmol/L)	1728 ± 108	1709 ± 70	1718 ± 61	2776 ± 127	2754 ± 188	2766 ± 107 ***
Plasma SM (μmol/L)	626 ± 47	609 ± 43	617 ± 31	943 ± 53	828 ± 65	891 ± 42 ***
Urinary choline (mg/g cr)	3.7 ± 1.3	3.8 ± 0.9	3.7 ± 0.8	20.5 ± 5.8	18.7 ± 5.4	19.7 ± 3.8 ***
Urinary betaine (mg/g cr)	8.8 ± 2.2	7.4 ± 1.3	8.1 ± 1.2	21 ± 6.1	13 ± 3.2	17.6 ± 3.8 *
Urinary DMG (mg/g cr)	4.0 ± 0.6	6.8 ± 1.1 [b]	5.5 ± 0.7	5.7 ± 1.4	6.8 ± 1.1	6.2 ± 0.9
Urinary sarcosine (mg/g cr)	0.45 ± 0.06	0.57 ± 0.06 #	0.5 ± 0.04	0.5 ± 0.06	1.2 ± 0.44 #	0.8 ± 0.22
Urinary methionine (mg/g cr)	3.0 ± 0.2	2.8 ± 0.3	2.9 ± 0.2	5.9 ± 2.0	4.9 ± 0.6	5.4 ± 1.1 *
Urinary homocysteine (mg/g cr)	0.45 ± 0.04	0.46 ± 0.06	0.46 ± 0.03	0.7 ± 0.06	1.2 ± 0.28	0.9 ± 0.14 **

[1]Data are presented as mean ± SEM.

[2]2- way ANOVA (pregnancy, choline intake, and their interactions) was used to compare the concentrations of choline metabolites and biochemical indices related to one-carbon metabolism between pregnant and nonpregnant women; *n* = 19-23 per group; # indicates $0.05 < P < 0.1$, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$

[3]1-way ANOVA (choline intake) was used to compare the concentrations of choline metabolites and biochemical indices related to one-carbon metabolism between choline intake groups for pregnant women or nonpregnant women, separately; wk 0 measures of concentrations were included as covariates in the tests; n = 9-12 per group; [#] indicates $0.05 < P < 0.1$, [a] indicates $P \leq 0.05$, [b] indicates $P \leq 0.01$, [c] indicates $P \leq 0.001$

Abbreviations used: DMG, dimethylglycine; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine

Effects of maternal choline intake and pregnancy on the isotopic enrichment and enrichment ratio of choline metabolites

At study-end, labeled choline metabolites were detected at acceptable signal-to-noise ratios in blood and urine samples including d3-choline, d9-choline, d3-betaine, d9-betaine, d3-DMG, d6-DMG, d3-sarcosine, d3-methionine, d3-SAM, d3-PC, d6-PC, d9-PC, and d9-SM. The blood and urinary enrichment of the same metabolite was highly correlated (Pearson's correlation coefficient $r \geq 0.7$, $P < 0.001$).

Main effect of pregnancy

At the study-end, pregnant women had lower plasma enrichment of d9-choline ($P = 0.02$), d6-DMG ($P < 0.001$), d3-PC ($P = 0.005$), d6-PC ($P = 0.002$), and d9-PC ($P = 0.07$) and lower urinary enrichment of d9-choline ($P < 0.001$), d9-betaine ($P < 0.001$), and d6-DMG ($P < 0.001$) (**Table 2**). The calculated hepatic enrichment of d3-SAM ($P < 0.001$) was also lower among pregnant versus nonpregnant women. The systemically lower enrichment of labeled choline metabolites (e.g., d9-choline) among pregnant women may arise from a greater dilution of the labeled d9-choline in the unlabeled choline (d0-choline) and phosphatidylcholine (d0-PC) pools, which were approximately 50% higher among third trimester pregnant women as compared to nonpregnant women [4].

In contrast, third trimester pregnant women (versus nonpregnant women) tended to have higher ($P \leq 0.06$) plasma and urinary enrichment of d3-choline, a derivative of d3-PC produced by the PEMT pathway. Pregnant women also had higher plasma/urinary enrichment of d3-betaine ($P \leq 0.013$) and d3-DMG ($P = 0.01$), which are generated when d3-choline is used as a methyl donor. In addition, the enrichment of serum d3-methionine was 31% higher ($P = 0.022$) among pregnant versus nonpregnant women possibly reflecting greater synthesis of methionine from the endogenously produced d3-choline. Finally, the enrichment of d3-choline, the enrichment ratio of d3-choline:d3-PC ($P = 0.001$), and the

enrichment ratio of d3-betaine:d3-choline ($P = 0.08$) were higher among pregnant versus nonpregnant women, suggesting a greater hydrolysis of PEMT-PC tofree choline, and an enhanced use of choline liberated from PEMT-PC for betaine synthesis in late pregnancy.

The enrichment of PEMT-PC increased to a similar extent among pregnant and nonpregnant women: the change in PEMT-PC enrichment did not differ ($P > 0.7$) between pregnant and nonpregnant women from wk 9 to wk 10 (pregnant versus nonpregnant: 1.2 ± 0.7 versus 1.28 ± 1.1) or from wk 10 to wk 12 (pregnant versus nonpregnant: 1.35 ± 1.0 versus 1.38 ± 0.8). Given that (i) the plasma PC pool is ~ 1.5 times greater among pregnant versus nonpregnant women; and (ii) enrichment of PEMT-PC changed to a similar extent among pregnant and nonpregnant women, these data imply greater use of choline derived methyl groups for PC production through the PEMT pathway among third trimester pregnant women. These data are consistent with an upregulation of PEMT activity by estrogen [17, 18] which rises during the second half of pregnancy [19, 20].

TABLE 2: Blood and urinary enrichment and the enrichment ratios of choline metabolites at study-end (wk 12) in third trimester pregnant and nonpregnant women consuming ~ 22% of 480 or 930 mg choline/d as *methyl*-d9-choline from study wk 6 to wk 12[1]

	Nonpregnant women, *n* = 21			Third trimester pregnant women, *n* = 23		
Variables	480 mg/d	930 mg/d [3]	All [2]	480 mg/d	930 mg/d [3]	All [2]
Maternal blood enrichment						
d9-choline	7.0 ± 0.2	8.5 ± 0.3 [b]	7.7 ± 0.3	6.4 ± 0.3	7.6 ± 0.4 [a]	6.9 ± 0.3 [*]
d9-betaine	8.0 ± 0.2	11 ± 0.5 [c]	9.6 ± 0.5	6.7 ± 0.3	8.4 ± 0.4 [b]	7.4 ± 0.3 [***]
d6-DMG	29 ± 0.7	37 ± 1.0 [c]	33 ± 1.0	25 ± 1.0	31 ± 2.0 [b]	27 ± 1.0 [***]
d3-choline	2.3 ± 0.2	2.9 ± 0.1 [b]	2.6 ± 0.1	2.4 ± 0.1	3.4 ± 0.1 [c]	2.8 ± 0.2 [#]
d3-betaine	1.5 ± 0.1	1.9 ± 0.1 [b]	1.7 ± 0.1	1.7 ± 0.1	2.4 ± 0.1 [c]	2.0 ± 0.1 [*]
d3-sarcosine [4]	22 ± 0.6	29 ± 0.9 [c]	25 ± 1	21 ± 1	27 ± 1 [b]	24 ± 1
d3-methionine [4]	0.49 ± 0.05	0.67 ± 0.08 [#]	0.58 ± 0.05	0.62 ± 0.07	0.93 ± 0.11 [b]	0.76 ± 0.07 [*]
d3-PC	1.03 ± 0.06	1.51 ± 0.09 [c]	1.27 ± 0.08	0.86 ± 0.05	1.25 ± 0.09 [c]	1.03 ± 0.07 [**]
d6-PC	0.18 ± 0.01	0.43 ± 0.03 [c]	0.31 ± 0.03	0.13 ± 0.02	0.31 ± 0.04 [c]	0.21 ± 0.03 [**]
d9-PC	8.6 ± 0.4	9.9 ± 0.4 [b]	9.3 ± 0.2	8.0 ± 0.4	9.2 ± 0.5 [#]	8.5 ± 0.3 [#]
d9-SM	8.7 ± 0.3	10.1 ± 0.3 [b]	9.4 ± 0.3	8.2 ± 0.4	9.4 ± 0.5 [#]	8.7 ± 0.3 [#]
Hepatic d3-SAM [5]	0.152 ± 0.01	0.222 ± 0.01 [c]	0.187 ± 0.01	0.132 ± 0.01	0.191 ± 0.01 [c]	0.157 ± 0.01 [***]
Maternal blood enrichment ratios						
d9-betaine : d9-choline	1.14 ± 0.03	1.33 ± 0.03 [c]	1.24 ± 0.03	1.05 ± 0.16	1.10 ± 0.28 [#]	1.07 ± 0.02 [***]
d9-PC : d9-choline	1.23 ± 0.02	1.17 ± 0.02 [#]	1.20 ± 0.02	1.24 ± 0.03	1.20 ± 0.02	1.22 ± 0.02
d9-SM : d9-PC	1.01 ± 0.01	1.02 ± 0.01	1.02 ± 0.01	1.02 ± 0.01	1.03 ± 0.01	1.02 ± 0.01
d9-betaine : d9-PC	0.93 ± 0.03	1.14 ± 0.04 [c]	1.04 ± 0.04	0.85 ± 0.02	0.92 ± 0.02 [b]	0.88 ± 0.02 [***]
d6-DMG : d9-betaine	3.72 ± 0.11	3.29 ± 0.11 [a]	3.51 ± 0.09	3.67 ± 0.08	3.66 ± 0.09	3.67 ± 0.06
d3-met : d3+d9-betaine	0.052 ± 0.01	0.051 ± 0.01	0.051 ± 0.01	0.073 ± 0.01	0.087 ± 0.01	0.079 ± 0.01 [***]
d3-sarcosine : d6-DMG	0.76 ± 0.03	0.77 ± 0.01	0.76 ± 0.02	0.86 ± 0.02	0.89 ± 0.02	0.87 ± 0.01 [***]
d3+d6-PC : d9-PC	0.14 ± 0.01	0.20 ± 0.01 [b]	0.17 ± 0.01	0.13 ± 0.01	0.17 ±0.01 [b]	0.15 ± 0.01 [#]
d3-choline : d3-PC	2.22 ± 0.11	1.92 ± 0.11 [a]	2.07 ± 0.07	2.80 ± 0.14	2.78 ± 0.15	2.79 ± 0.10 [***]
d3-betaine : d3-choline	0.69 ± 0.02	0.67 ± 0.01	0.67 ± 0.01	0.71 ± 0.01	0.70 ± 0.01	0.70 ± 0.01 [#]

Urinary enrichment						
d9-choline	12.0 ± 0.8	14.3 ± 0.9 [#]	13.2 ± 0.6	9.0 ±0.7	11.3 ± 0.7 [a]	10.0 ± 0.5 [***]
d9-betaine	10.7 ± 0.5	13.6 ± 0.6 [c]	12.1 ± 0.5	7.9 ± 0.6	9.3 ± 0.4 [#]	8.5 ± 0.4 [***]
d6-DMG	35 ± 1.2	41 ± 0.9 [b]	38 ± 1.0	27 ± 1.5	32 ± 1.5 [a]	29 ± 1.2 [***]
d3-choline	2.2 ± 0.1	2.6 ± 0.2 [a]	2.4 ± 0.1	2.4 ± 0.1	3.0 ± 0.2 [b]	2.6 ± 0.1 [#]
d3-betaine	1.6 ± 0.1	1.8 ± 0.1 [a]	1.7 ± 0.1	1.7 ± 0.1	2.3 ± 0.1 [b]	2.0 ± 0.1 [*]
d3-DMG	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1 [**]
d3-sarcosine	23 ± 2.3	30 ± 1.9 [a]	27 ± 1.6	21 ± 2.4	29 ± 1.8 [a]	25 ± 1.7
d3-met	0.44 ± .07	0.77 ± 0.08 [b]	0.60 ± 0.07	0.55 ± 0.08	0.65 ± 0.05	0.59 ± 0.05
d3-SAM	0.58 ± 0.03	0.72 ± 0.05 [a]	0.65 ± 0.03	0.49 ± 0.03	0.70 ± 0.04 [c]	0.58 ± 0.03
Urinary enrichment ratios						
d9-betaine : d9-choline	0.89 ± 0.02	0.96 ± 0.04	0.93 ± 0.02	0.87 ± 0.02	0.84 ± 0.04	0.86 ± 0.02 [*]
d3-betaine : d3-choline	0.72 ± 0.01	0.71 ± 0.02	0.72 ± 0.01	0.73 ± 0.01	0.75 ± 0.01	0.74 ± 0.01 [*]
d6-DMG:d9-betaine	3.4 ± 0.18	3.1 ± 0.13	3.2 ± 0.11	3.53 ± 0.16	3.39 ± 0.09	3.5 ± 0.10
d3-DMG:d3-betaine	0.84 ± 0.02	0.75 ± 0.02 [b]	0.79 ± 0.02	0.85 ± 0.03	0.76 ± 0.02 [a]	0.81 ± 0.02
d3-met : d3+d9-betaine	0.037 ± 0.007	0.050 ± 0.006	0.044 ± 0.005	0.061 ± 0.01	0.056 ± 0.004	0.059 ± 0.006 [*]
d3-sarcosine : d3+6-DMG	0.65 ± 0.07	0.70 ± 0.04	0.67 ± 0.04	0.74 ± 0.08	0.86 ± 0.05	0.79 ± 0.05 [#]
d3-SAM : d3-methionine	1.63 ± 0.24	1.08 ± 0.14 [#]	1.37 ± 0.15	1.10 ± 0.16	1.13 ± 0.12	1.11 ± 0.10

[1]Data are presented as mean ± SEM.

[2]2- way ANOVA (pregnancy, choline intake, and their interactions) was used to compare the enrichment or enrichment ratio between pregnant and nonpregnant women; n = 19-23 per group; [#] indicates $0.05 < P < 0.1$, [*] indicates $P \leq 0.05$, [**] indicates $P \leq 0.01$, [***] indicates $P \leq 0.001$

[3]1-way ANOVA (choline intake) was used to compare the enrichment or enrichment ratio between choline intake groups for pregnant women or nonpregnant women, separately; n = 9-12 per group; [#] indicates $0.05 < P < 0.1$, [a] indicates $P \leq 0.05$, [b] indicates $P \leq 0.01$, [c] indicates $P \leq 0.001$

[4] Methionine and sarcosine enrichment were obtained in serum whereas the concentrations of other metabolites were obtained in plasma.

[5] Hepatic enrichment of d3-SAM was calculated using the plasma enrichment of d3-PC and d6-PC according to the principle of mass isotopomer distribution analysis (MIDA) using the equation: hepatic d3-SAM enrichment = d6-PC enrichment/ (d3-PC enrichment + d6-PC enrichment)

Abbreviations used: DMG, dimethylglycine; met, methionine; SAM, *S*-adenosylmethionine; PC, phosphatidylcholine; SM, sphingomyelin

Pregnancy x choline intake interaction

Pregnancy interacted with choline intake to affect plasma enrichment of d9-betaine ($P = 0.034$). Plasma d9-betaine enrichment was 16% lower ($P = 0.003$) among pregnant versus nonpregnant women consuming 480 mg choline/d and was 25% lower ($P < 0.001$) among pregnant versus nonpregnant women consuming 930 mg choline/d (Table 2). In addition, the enrichment ratio of d9-betaine:d9-PC was 10% lower ($P = 0.022$) among pregnant versus nonpregnant women consuming 480 mg choline/d and was 19% lower ($P < 0.001$) among pregnant versus nonpregnant women consuming 930 mg choline/d. These data suggest a reduced partitioning of choline to betaine synthesis relative to the CDP-choline pathway in late pregnancy. In addition, the greater difference (19% versus 10% for d9-betaine:d9-PC) at the 930 versus 490 mg choline/d intake level is consistent with greater oxidation of choline by nonpregnant (versus pregnant) women on the higher choline intake. Notably, the enrichment ratio of d9-betaine:d9-PC in third trimester pregnant women consuming 930 mg choline/d was not different from that of nonpregnant women consuming 480 mg choline/d (0.92 ± 0.02 vs. 0.93 ± 0.03; $P = 0.9$) indicating that a higher choline intake is needed by third trimester pregnant women to ensure an adequate supply of betaine under conditions of enhanced PC production by CDP-choline pathway.

Main effect of choline intake

As expected, a higher enrichment of almost all the choline metabolites was observed in biological samples collected from pregnant and nonpregnant women consuming 930 versus 480 mg choline/d (**Table 2 and 3).**

Among both pregnant and nonpregnant women, plasma enrichment ratios of d9-betaine:d9-choline, d9-betaine:d9-PC, and (d3+d6)-PC:d9-PC were higher in the 930 versus 480 mg choline/d intake group, suggesting that a higher choline intake favors oxidation of choline and the use of choline derived methyl groups for PEMT mediated PC synthesis.

Among nonpregnant women, consumption of 930 (versus 480) mg choline/d yielded a greater (P = 0.033) change of the enrichment of PEMT-PC (d3+d6-PC) between wk 10 and wk 12, compared to 480 mg choline/d (1.7 ± 0.8 vs. 1.0 ± 0.6), suggesting that a higher choline intake increases PC production through PEMT pathway in nonpregnant women. Because PC synthesized through PEMT pathway is enriched with DHA, this finding is consistent with our previous observation that the proportion of PC-DHA in total PC molecule was higher in nonpregnant women consuming 930 versus 480 mg choline/d (West et al. submitted). No effect of choline intake on the change of the enrichment of PEMT-PC (d3+d6-PC) was detected among pregnant women.

It is interesting to note that among nonpregnant women, the plasma enrichment ratio of d6-DMG:d9-betaine (P = 0.016), the urinary enrichment ratio of d3-SAM:d3-methionine (P = 0.07), d3-DMG:d3-betaine (P = 0.003), and the plasma enrichment ratio of d3-choline:d3-PC (P = 0.028) were lower in the 930 vs. 480 mg choline/d intake group. These data suggest that although the higher choline intake increased the use of choline as a methyl donor, segments throughout the oxidative pathway were affected to a different extent.

TABLE 3: Enrichment of choline metabolites in delivery blood, placental tissue, and fetal cord blood obtained from pregnant women consuming ~22% of 480 or 930 mg choline/d as *methyl*-d9-choline from study wk 6 till delivery [1,2]

Variables	Choline Intake	
	480 mg/d, n = 12	930 mg/d, n = 12
Maternal delivery blood		
d9-choline	4.9 ± 0.5	6.6 ± 0.6 [a]
d9-betaine	5.9 ± 0.6	8.4 ± 0.8 [a]
d6-DMG	14.5 ± 0.9	19.4 ± 1.3 [b]
d3-choline	0.58 ± 0.07	0.87 ± 0.11 [a]
d3-PC	2.0 ± 0.2	2.8 ± 0.2 [b]
d6-PC	0.12 ± 0.02	0.28 ± 0.03 [c]
d9-PC	7.1 ± 0.7	8.8 ± 0.8
d9-SM	7.8 ± 0.6	9.1 ± 0.7
Placental tissue		
d9-choline	3.3 ± 0.3	4.4 ± 0.4 [a]
d9-betaine	4.6 ± 0.4	6.4 ± 0.5 [a]
d3-choline	1.0 ± 0.1	1.5 ± 0.1 [b]
d3-PC	2.2 ± 0.2	2.9 ± 0.2 [a]
d9-PC	3.9 ± 0.4	4.9 ± 0.5 [#]
d9-SM	5.0 ± 0.4	5.7 ± 0.5
Fetal cord blood		
d9-choline	3.6 ± 0.3	4.5 ± 0.4
d9-betaine	4.6 ± 0.4	5.8 ± 0.4 [a]
d6-DMG	16.5 ± 1.7	19.4 ± 1.5
d3-choline	1.8 ± 0.2	2.7 ± 0.2 [b]
d3-PC	3.6 ± 0.2	4.5 ± 0.2 [b]
d9-PC	3.0 ± 0.2	3.8 ± 0.3 [#]
d9-SM	3.7 ± 0.3	4.3 ± 0.3 [#]

[1]Data are presented as mean ± SEM.

[2]Data were analyzed with 1-way ANOVA (choline intake); n = 9-12 per group; [#] indicates $0.05 < P < 0.1$, [a] indicates $P \leq 0.05$, [b] indicates $P \leq 0.01$, [c] indicates $P \leq 0.001$

No effect of choline intake on the enrichment ratios within each compartment was detected except that the enrichment ratio of d9-betaine:d9-PC was higher in the 930 versus 480 mg choline/d intake group in maternal delivery blood (0.96 ± 0.02 versus 0.83 ± 0.04, P = 0.014).

Abbreviations used: DMG, dimethylglycine; PC, phosphatidylcholine; SM, sphingomyelin.

Comparisons of the enrichment of choline metabolites among delivery blood, placenta, and cord blood

As presented in the **Figure 2**, the enrichment of d3-choline and d3-PC in the three compartments was in the order of maternal delivery blood < placental tissue < fetal cord blood ($P < 0.001$ for any comparisons between two compartments). In contrast, the enrichment of d9-PC and d9-SM was in the reverse order of maternal delivery blood > placental tissue > fetal cord blood ($P < 0.001$ for any comparisons between two compartments).

It is also notable that the enrichment of d6-DMG tended to be higher in the cord blood compared to maternal blood ($P = 0.06$). Because DMG is not transferred from the maternal to fetal compartment [4], these data imply an active use of choline/betaine derived methyl groups in fetal compartment and are consistent with enhanced BHMT activity in human infants [21, 22].

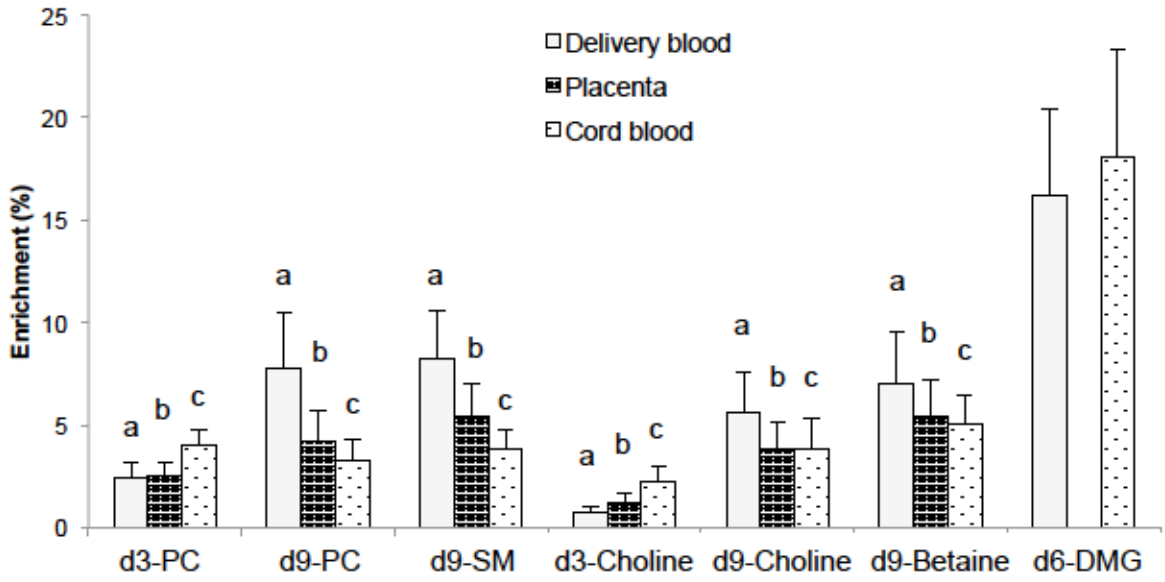

Figure 2 legend

Enrichment of choline metabolites in delivery blood, placental tissue, and fetal cord blood obtained from pregnant women consuming ~ 22% of 480 or 930 mg choline/d as *methyl*-d9-choline from study wk-6 till delivery. Paired t tests were conducted in comparing the enrichment of corresponding choline metabolite among three compartments. Different letters (a, b, c) indicate differences between compartments at *P-values* < 0.001. Similar results were obtained when the analyses were performed separately for each choline intake group. Abbreviations: DMG, dimethylglycine; PC, phosphatidylcholine; SM, sphingomyelin

DISCUSSION

This study employed stable isotope methodology to investigate the effect of pregnancy and choline intake on the metabolic use of orally consumed choline in humans. Three main findings emerged: (i) pregnancy increases the production of PC through both the CDP-choline and PEMT pathways; (ii) PEMT-PC is selectively transported to the fetus; and (iii) a choline intake exceeding current recommendations partially restores the pregnancy-induced alterations in choline metabolism.

Effect of pregnancy on choline metabolism

Pregnancy increases choline partitioning to the CDP-choline pathway at the expense of betaine synthesis

Pregnant (versus nonpregnant) women used more choline for PC synthesis through the CDP-choline pathway (**Figure 3**) as indicated by a lower enrichment ratio of d9-betaine:d9-PC. The enhanced use of choline for PC production via the CDP-choline pathway is due in part to the hepatic lipid load experienced by women during late pregnancy [23]. Specifically, PC is required for the biosynthesis of very low-density lipoproteins (VLDL), which mediate the export of lipid from liver into circulation. Pregnant (versus nonpregnant) women also exhibited a lower d9-betaine:d9-choline enrichment ratio as well as diminished circulating concentrations of metabolites produced in the oxidative pathway including betaine, dimethylglycine, sarcosine, and methionine [4]. Thus, in the face of enhanced use of choline for PC production by the CDP-choline pathway, third trimester pregnant women require substantially more choline than their nonpregnant counterparts to maintain an adequate supply of choline derived methyl groups for one-carbon metabolism.

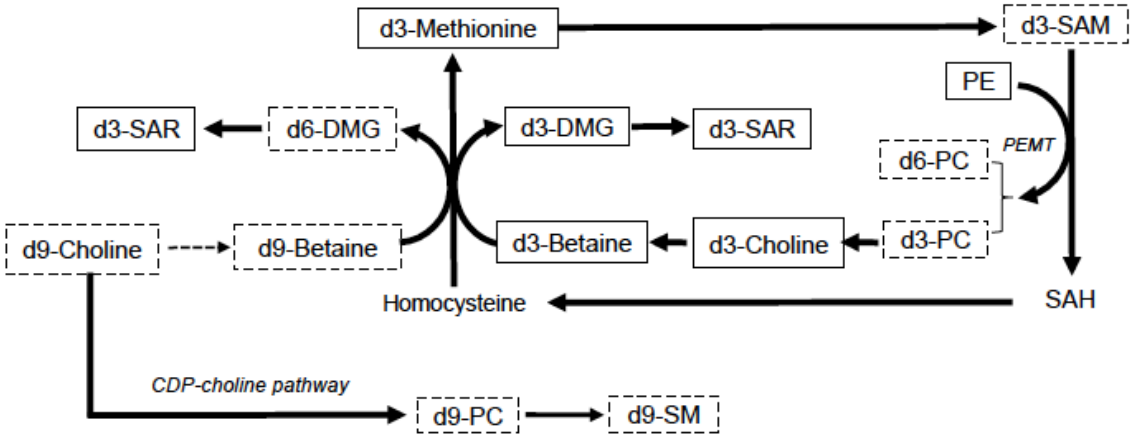

Figure 3 legend

A working hypothesis of pregnancy induced alterations in choline metabolic pathways. Pregnancy reduces the use of orally consumed choline for betaine synthesis, but enhances the use of choline for PC synthesis through CDP-choline pathway, enhances the use of betaine for methionine synthesis and PC synthesis through PEMT pathway, and enhances the use of endogenously produced choline as a methyl donor.

Boxed (solid and dashed) metabolites are those whose enrichment were quantified in the study; solid box, the metabolite enrichment higher or similar in pregnant compared to nonpregnant women; dashed box, the metabolite enrichment lower in pregnant compared to nonpregnant women.

Thick arrow, metabolic flux enhanced in third trimester pregnant versus nonpregnant women; dashed arrow, metabolic flux attenuated in third trimester pregnant versus nonpregnant women.

Abbreviations: DMG, dimethylglycine; SAR, sarcosine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SM, sphingomyelin; PEMT, phosphatidylethanolamine N-methyltransferase.

Pregnancy increases the use of choline derived methyl groups for PC synthesis through PEMT pathway

Despite an enhanced partitioning of orally consumed choline to the CDP-choline pathway during late pregnancy, the use of choline derived methyl groups for PC synthesis through the PEMT pathway was elevated in pregnant versus nonpregnant women (Figure 3). Specifically, the incremental labeling of the plasma PC pool with PEMT-PC did not differ between pregnant and nonpregnant women. As the PC pool size is approximately 50% greater among pregnant versus nonpregnant women, these data indicate that flux of choline derived methyl groups through the PEMT pathway is enhanced during late pregnancy. The high demand for methyl groups by the PEMT pathway during pregnancy is consistent with its upregulation by estrogen [17, 18] which rises during the second half of gestation [19, 20].

It is also noteworthy that the enrichment of d3-choline, the hydrolysis product of PEMT-PC, was higher in pregnant versus nonpregnant women. The higher enrichment of d3-choline along with the higher enrichment ratio of d3-choline:d3-PC in pregnant versus nonpregnant women suggests that pregnant women catabolize more PEMT-PC in order to generate free choline. The enhanced hydrolysis of PEMT-PC may explain the rise in plasma free choline during the second half of gestation. Whether the hydrolysis of PC derived from the CDP-choline pathway contributes to the elevation of plasma free choline concentration during late pregnancy cannot be determined as the labeling strategy used in the present study does not distinguish between orally consumed d9-choline and d9-choline liberated from d9-PC.

Pregnancy increases the use of choline derived methyl groups for methionine biosynthesis

Although less betaine was synthesized from choline in pregnant versus nonpregnant women (i.e. lower d9-betaine:d9-choline enrichment ratio in pregnant women), more betaine was utilized for methionine synthesis in pregnant women (Figure 3). Specifically, d3-methionine enrichment was elevated among pregnant (versus nonpregnant) women as was the enrichment ratio of d3-methionine:(d3+d9)-betaine. Thus, the 50% lower plasma betaine concentration observed in late pregnancy likely arises from both reduced betaine synthesis and enhanced utilization of betaine for methionine production.

PEMT-PC is selectively directed towards the fetus

The stepwise increase in d3-PC enrichment, but not d9-PC enrichment, from the maternal to the fetal compartment implies that PEMT-PC is selectively directed towards the fetus. This partitioning of PEMT-PC towards the fetus may be due to its enrichment with DHA. Specifically, compared to the CDP-choline pathway, the PEMT pathway produces a PC molecule that is enriched in DHA [15, 24, 25], a long chain unsaturated fatty acid that is critical for fetal brain development [26, 27]. Once made, the PC-DHA molecule can be incorporated into VLDL and exported into circulation. The circulating PC-DHA molecule is subsequently available to the peripheral tissues including the placenta and developing fetus.

A choline intake exceeding current recommendations may be needed to support the demands of pregnancy

The higher choline intake (930 versus 480 mg/d) among pregnant women restored the partitioning of choline between the CDP-choline and choline oxidative pathways to the nonpregnant state. Specifically, a doubling of maternal choline intake during the later part of pregnancy yielded a d9-betaine:d9-PC enrichment ratio that did not differ ($P = 0.9$) from nonpregnant women consuming 480 mg choline/d. In addition, consumption of 930 mg choline/d among pregnant women elevated their plasma betaine concentrations to a level that

was closer to that of nonpregnant women consuming 480 mg choline/d [4]. These data suggest that a choline intake exceeding current recommendations may be needed to support both PC production via the CDP-choline pathway and choline mediated one-carbon metabolism.

In addition, a higher choline intake enhanced the use of choline as a methyl donor for PC synthesis via the PEMT pathway among both pregnant and nonpregnant women. Specifically, consumption of 930 versus 480 mg choline/d yielded higher enrichment ratios of d9-betaine:d9-choline, (d3+d6)-PC: d9-choline, and (d3+d6)-PC:d9-PC independent of reproductive state. Moreover, a higher choline intake stimulated PC production through the PEMT pathway among nonpregnant women. Specifically, the incremental labeling of the plasma PC pool with PEMT-PC was greater among nonpregnant women consuming 930 versus 480 mg choline/d. The up-regulated activity of the PEMT pathway among third trimester pregnant women (versus nonpregnant women) may have precluded the stimulatory effect of choline on PEMT activity during late pregnancy. Collectively these data suggest that a higher choline intake may stimulate the PEMT pathway and thus mobilize PC-DHA to peripheral tissues among nonpregnant women and presumably among pregnant women at an earlier stage in gestation.

CONCLUSION AND IMPLICATIONS

The enhanced use of choline for PC production via both the CDP-choline and PEMT pathways demonstrates the substantial demand for choline during late pregnancy. Our finding that a choline intake exceeding current recommendations restores the partitioning of choline between the CDP-choline pathway and betaine synthesis pathways among pregnant women (as compared to nonpregnant women consuming 480 mg choline/d) suggests that current recommendations may be suboptimal during gestation. The selective partitioning of PEMT-PC to the fetal compartment may imply a unique requirement of PEMT-PC for the developing fetus.

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AFTERWORD

MTHFR 677TT genotype increases the use of choline as a methyl donor

Genetic variation can modify disease risk and more than 800 human studies examining the relationship between *MTHFR* C677T genotype and disease risk have been published since its discovery in 1995 [1]. The 677TT genotype has been shown to increase the risk of cardiovascular disease, neural tube defects, cognitive impairments and certain types of cancer [2, 3]. Nevertheless, it is well established that a high folate intake can abolish the adverse effects of the *MTHFR* 677TT genotype [4] thereby illustrating the importance of personalized nutrition, which bases nutrient recommendations on an individual's genetic profile. The findings of my dissertation work demonstrate that choline may also be useful in mitigating the adverse effects of the *MTHFR* 677TT genotype. Specifically, in the face of impaired ability to produce 5-methyl-THF (the product of MTHFR), individuals with the *MTHFR* 677TT genotype used more choline as a methyl donor. Moreover, a choline intake level exceeding current recommendations increased the use of choline derived methyl groups only in the 677TT but not 677CC genotype. This unique ability to respond to extra choline among those with the *MTHFR* 677TT genotype coupled with their greater reliance on choline as a methyl donor implies that a choline intake exceeding current recommendations may improve health outcomes among this genetic sub-group.

A maternal choline intake exceeding current recommendations increases the use of choline as a methyl donor in the maternal and fetal compartments

Pregnancy is associated with a higher demand for choline due to accelerated 1-C metabolism and the formation of new membranes as cells undergo division [5]. Pregnancy causes a pronounced reduction of choline pools in rodents consuming a normal chow diet [6], indicating that the need for this nutrient by the mother and the fetus may exceed the amount

consumed by the mother and the amount produced by the endogenous pathway (i.e., phosphatidylethanolamine *N*-methyltransferase pathway). Moreover, supplementing the maternal rat diet with additional choline (approximately 4 times the amount in normal chow) leads to a significant enhancement of memory function of the adult offspring and substantially lessens aging-related memory decline [7-10]. The lasting beneficial effects of maternal supplemental choline on cognitive functioning in the adult offspring are mediated in part by alterations in genomic DNA/histone methylation [11, 12].

The findings of my dissertation document depletion of choline derived methyl donors among third trimester pregnancy women as well as enhanced biosynthesis of PC through both the CDP-choline and denovo pathways. Notably, consumption of a higher choline intake partially restores these pregnancy induced metabolic alterations and leads to greater use of choline as a methyl donor in both the maternal and fetal compartment. The implications of the effects of a higher maternal choline intake during late pregnancy have been elucidated in separate research publications by our research group [13-14]. For example, a higher maternal choline intake alters the methylation of cortisol regulating genes resulting in lower circulating concentrations of the stress hormone, cortisol [13]. Collectively, the study findings suggest that consuming extra choline during pregnancy increases the availability of choline derived methyl groups with downstream effects on "programming" baby's responsiveness to stress.

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